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Table: -, Figs : 5

Effects of tricin isolated from jungle rice (*Echinochloa colona* L.) on amylase activity and oxidative stress in wild oat (*Avena fatua* L.)

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ABSTRACT

Bioactive compounds were isolated from the jungle rice (*Echinochloa colona* L.) and their phytotoxicity was evaluated against the weed wild oat (*Avena fatua* L.). Tricin (flavonoid compound) purified from *E. colona* was tested at 5, 25, 50 and 100 µM on wild oat growth. Tricin suppressed the germination and growth of target weed at higher concentrations (25, 50 and 100 µM). The inhibitory effects of tricin were due to the attenuation of amylase activity, which decreased the starch mobilization and also hampered the activities of antioxidant enzymes. The increase in the non-enzymatic antioxidant molecules (polyphenol, ascorbate and glutathione) was not sufficient to prevent the damage from tricin, because the level of H_2O_2 and lipid peroxidation was high. More studies are required to evaluate the use of tricin as bioherbicide to control the wild oat.

Key words: Antioxidants, *Avena fatua* L., *Echinochloa colona* L., enzymes, jungle rice, organic fractions, oxidative stress, phytotoxicity, tricin, wild oat.

INTRODUCTION

Recently, great attention has been paid to discover the compounds with new sites of action (8,14,18) and are environmental friendly. Crop plants have to defend themselves from pest attacks; they also face competition with other plants for soil nutrients and other resources. Higher plants, algae, fungi or microorganisms produce active secondary metabolites, which effects other plants or microbial community**.** The plants release these chemicals through leaching, exudation, volatilization or decomposition of biomass. Some of these compounds alters the growth or physiological functions of other organisms (15,38,61). For example, barnyardgrass (*Echinochloa crus-galli* L.) roots exude *p*-hydroxymandelic acid, which is growth-inhibitory allelochemical (58). Additionally, the action of released bioactive metabolites cause alterations at morphological, cellular or molecular level of receiver plants (49,61). Some phytotoxins may effectively control the weeds (21), thereby decreasing the reliance on synthetic herbicides and improving the ecological environment (24,34). For instance, some bioactive compounds isolated from weeds [parthenin from *Parthenium hysterophorus* L. (2) and artemisinin from *Artemisia* sp.] are used as bioherbicides (13,23).

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Many categories of bioactive compounds (phenolics, sesquiterpenes, flavonoids, terpenes, alkaloids) are very phytotoxic against various weeds (43). Flavonoids are highly active and may be associated with crop resistance to weeds (9,30,50), besides some flavonoids extracted from weeds at certain concentrations inhibited the growth of other weeds (43). Tricin is present in gramineae plants [*Echinochloa utilis* (54), *Avena sativa* (55), *Pleioblastus amarus*, (53), *Poa hoecu* (44), *Oryza sativa* (5), wild and cultivated wheat (*Triticum*, *Aegilops*, *Hordeum*) (11)] and provides plant defense against weeds and fungi (30). It also possesses herbicidal activity (9) and its novel flavonolignan acts as germination inhibitor (10).

This study aimed to isolate the most bioactive compounds from jungle rice and to test its phytotoxicity against wild oat (most widespread and harmful weed) (12,46).

MATERIAL AND METHODS

Jungle rice samples were collected from a maize field at the flowering stage and were separated into shoots and roots. These were dried at room temperature for 2-weeks and then ground to fine powder (2 mm size) using a grinder. About 2 kg fine powdered shoots were used for chromatographic analyses. Seeds of wild oat were collected in 2008 from infected wheat fields in Beni-Suef (arid climate). Healthy seeds were selected, cleaned and kept in polyethylene bags for further use.

Extraction and isolation of bioactive compounds: Dried shoots of jungle rice were extracted by soaking with methanol and the extract was separated sequentially with heptane, dichloromethane, butanol and finally with aqueous ethanol. Dichloromethane (CH_2Cl_2) fraction was the most active fraction when tested on a lettuce seed bioassay, and tricin was isolated from this fraction. Further separation of CH_2Cl_2 fraction on a silica gel column chromatography (Merck KGaA, Darmstadt, Germany) was done using a gradient of acetone: ethanol (30:1 to 5:1) as a mobile phase depending upon thin layer chromatography (TLC) analysis (Merck KGaA, Darmstadt, Germany) to obtain 12 sub-fractions. The sub-fractions were subjected to another bioassay for lettuce seeds germination to determine the most active sub-fraction (F2-9) which then was concentrated under pressure using rotary evaporator. The concentrated sub-fraction (F2-9) was eluted on Sephadex LH-20 chromatography (Merck KGaA, Darmstadt, Germany) by using CH_2Cl_2: methanol (20:1 to 10:1) then the elute was divided in to 4- sub-fractions (F2-9-1 to F2-9-4). The sub-fraction F2-9-4 was found the most active than other sub-fractions, which was then concentrated under reduced pressure and purified by HPLC with ODS column to yield a pure compound. The isolated pure compound was identified based on spectroscopic analyses (^1H and ^{13}C NMR).The detailed procedure regarding the extraction and chromatographic isolation has been described by Gomaa and AbdElgawad (23).

Structure analysis of isolated active compound: The pure compound was identified based on spectroscopic analyses (^1H and ^{13}C NMR) [^1H NMR (DMSO-d6) δ; 3.88 (6H, s, CH_3O- × 2), 6.20 (^1H, d, J =2.0 Hz, H-6), 6.55 (^1H, d, J = 2.0 Hz, H-8), 6.93 (^1H, s, H-3), 7.31 (2H, s, H-2'and 6') and ^{13}C NMR (DMSO-d6) δ; 56.4 (6H, s, CH_3O- × 2), 94.1 (C-8), 98.3 (C-6), 103.9 (C-3), 105.6 (C-10), 104.6 (2C, C-2' and 6'), 120.2 (C-1'), 139.9 (C-4'),

148.1 (2C, C-3' and 5'), 156.9 (C-9), 157.7 (C-5), 163.6 (C-2), 164.2 (C-7), 182.1 (C-4)]. The spectrometry analyses results were compared with previous literature, which indicated the compound as tricin (23,31,49,54).

Lettuce seed bioassay: Lettuce (*Lactuca sativa* L.) seeds were used to test the germination response to different concentrations of fractions and sub-fractions collected during column chromatography. A very little amount of dimethyl sulfoxide (DMSO) was used to redissolve the dry fractions and sub-fractions, then diluted by distilled water to 5-concentrations (25, 50, 100, 200 and 400 mg L^{-1}). All seeds were surface sterilized with 0.1% mercuric chloride for 5-min then washed thrice in distilled water. Three replicates, each of 50 seeds, were prepared for each treatment using sterile Petri dishes (4 cm dia) lined with one sterile filter paper (Whatman Number 1). Five ml of test solutions were added to each Petri dish. The control groups were treated with 5- ml of 1% DMSO. Prepared Petri dishes were then placed in incubator in dark at 30 °C (NO: G150, Biotech Co. for Medical & Laboratory Equipments, Cairo, Egypt). After 5- days, the seedling length was measured to know the sensitivity of lettuce seeds to test fractions and sub-fractions.

Tricin bioassay: Tricin was dissolved in little amount of DMSO to prepare the highest test concentration (100 µM). It was further diluted to obtain 5, 25 and 50 µM concentrations. Distilled water with 1% of DMSO was used as control. Ten mL of each concentration was added into filter papers (Whatman, number 1) in 12 cm Petri dishes as per treatments. Then 50-healthy wild oat seeds were sown on filter paper. The Petri dishes were incubated at 25°C in dark for 10-days. Then germination (%) and length of radicals and plumules were recorded.

Metabolite analysis: Total soluble sugars and total starch were extracted as per methods of Upmeyer and Koller (51) and determined based on the anthrone-sulfuric acid assay (59), phenolic was extracted (26) and estimated by Folin-Ciocalteau phenol reaction (1). Total glutathione content (tGSH) was estimated by modified Griffith (24) procedure and total ascorbate (tASC) was assayed by Kampfenkel et al. (27).

Enzyme activities: Amylase enzyme was extracted (36) and its activity was assayed as per Bilderback (4). Fresh seedlings of wild oat were extracted with 2.5 mL of 67 mM cold phosphate buffer (pH 7.0) as per Shann and Blum (47). The homogenates were centrifuged at 10,000 rpm for 15 min at 4ºC. The clear supernatant was used as a raw extract material for enzymatic assay. Catalase (CAT) (EC 1.11.1.6), peroxidase (POX) (EC 1.11.1.7) and polyphenol oxidase (POL) (EC 1.10.3.1) activity was measured as per Kar and Mishra (48). CAT activity was assayed by measuring the decline in H_2O_2 absorbance at 240 nm. The activity was calculated using the extinction coefficient (40 mM^{-1} cm^{-1} at 240 nm) and expressed in units of µM of destroyed H_2O_2 min^{-1} g^{-1} fresh weight. POX and POL 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 as per the method of Beyer and Fridovich (3). One unit of SOD activity was defined as the amount of enzyme required to cause inhibition of the photo-reduction of nitro blue tetrazolium (NBT) by 50% (U mg^{-1} FW).

H_2O_2 and Lipid Peroxidation (MDA): H_2O_2 content was measured colorimetrically and levels were calculated using the extinction coefficient 0.28 μmol^{-c} cm^{-c} (62). Lipid peroxidation (MDA) was estimated by measuring the concentration of thiobarbituric acid reactive substances (TBARS) and expressed as nmol MDA g^{-1} FW using the extinction coefficient (156 mM^{-1} cm^{-1}) (42).

Statistical analysis: All experiments data were statistically analysed by one way ANOVA using SPSS 16.0 statistical software and significant differences between the means of the parameters (n=5) were determined by using Duncan test ($P < 0.05$).

RESULTS AND DISCUSSION

Investigation of fractions activity

In lettuce seed bioassay, among the different organic solvent fractions and subfractions of jungle rice, CH_2Cl_2 fraction and its two sub-fractions, F2-9 and F2-9-4 had the highest bioactivity resulted in maximum growth inhibition (61, 47 and 41%, respectively) (data not shown). The subfraction F2-9-4 was chromatographed using HPLC with ODS column to yield the sufficient pure compound (95.3 mg).

Germination, seedling growth and Dry Weight

The lower concentration (5 µM) of extracted tricin did not effect the germination and growth parameters but the higher concentrations (25, 50 and 100 µM) reduced the wild oat germination (Fig. 1a), growth (Fig. 1b) and biomass production (Fig. 1c). Similarly, exogenous application of flavonoids reduced the growth of some gramineous plants [(*Oryza sativa* L. cv. Koshihikari), maize (*Zea mays* L. cv. Yellow corn) and *Echinochloa oryzicola*] (16). The alterations in the plant growth were concentration dependent of applied bioactive compounds (22,33). We found that tricin was highly phytotoxic at 100 µM and caused maximum reduction in germination (88%), radicle (93.7%) and plumule lengths (75.4%). Tricin is highly phytotoxic to growth of some weeds (*Echinochloa crus-galli*, *Cyperus iris* and *Cyperus difformis*) (30).

In our studies, the inhibitory effects were proportional to tricin concentration as reported by Nandakumar and Rangaswamy (37). Moreover, radicle growth was more affected than the plumule growth. The young seedlings, especially the roots are very sensitive to phytotoxic agents than adult plants or other plant organs (7,51). The variability in root and shoot sensitivity was because the roots were in direct contact with the extract and subsequently with inhibitory chemicals.

α-Amylase activity and Sugars

Tricin inhibited the α-amylase activity at higher concentrations (50 and 100 µM (53.8 and 77.9 %,) (Fig. 2a) and thereby decreased the total soluble sugars and starch accumulation (Fig. 2b). Similarly, Podesta and Plaxton (40) recorded the suppression of amylase activity in maize seeds and seedlings by using phenolic compounds. Amylase inhibition lead to the accumulation of starch content and reducing the content of total soluble sugars in wild oat, because during germination the amylase hydrolyses the starch

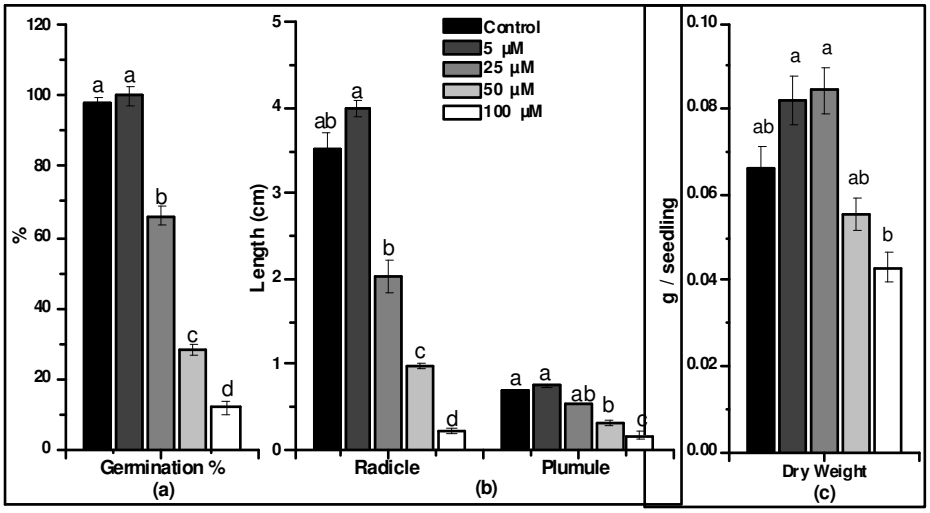

Figure 1. The inhibitory/stimulatory effects of tricin on (a) germination and (b) seedling growth of treated wild oat seedlings 10 days after sowing. Values are means of 5 replicates ± SE. Values with similar letters are not significantly different at p =0.05.

to soluble sugars (39). Furthermore, the accumulation of starch carbohydrates in treated wild oat plants may be ascribed to the interference of tricin with respiration rate. In this respect, the higher concentrations of flavonoids influenced the respiration rate through inhibition of mitochondrial oxygen uptake (20). Our data suggest that tricin decreased the α-amylase activity leading to suppression of starch mobilization and consequently decreasing the germination and growth of wild oat.

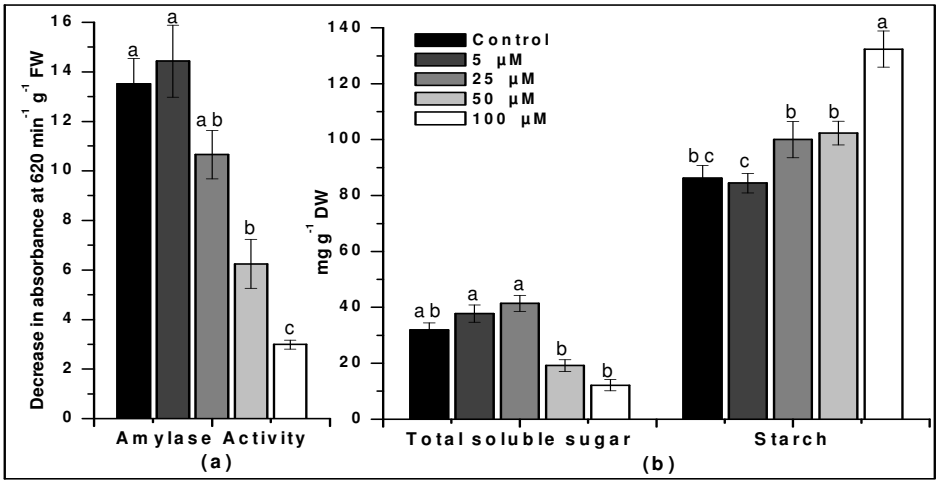

Figure 2. The effects of tricin concentrations on (a) amylase activity and (b) total soluble and starch contents of treated wild oat seedlings 10 days after sowing. Values are means of 5 replicates ± SE. Values with similar letters are not significantly different at p =0.05.

H_2O_2 accumulation enhanced lipid peroxidation (MDA) in response to tricin

The lowest concentration of tricin (5 µM) slightly decreased the level of H_2O_2 and lipid peroxidation. But its phytotoxic concentrations (50 and 100 µM) markedly accumulated the H_2O_2 (32.6 and 91.8%), instigating lipid peroxidation of membranes (25.4 and 80.4%) (Fig. 3). Our results agreed with Treutter (50) who reported that flavonoids increased the reactive oxygen species, which leads to Ca^{2+} signalling cascade causing the death of root system. The excessive ROS causes the lipid peroxidation when superoxide dismutase (SOD) and peroxidase (POX) are inhibited (6,35,49). Besides the reduction in the non-enzymatic antioxidant molecules (i.e. polyphenols, glutathione and ascorbate) may decrease the ability of plant to scavenge H_2O_2 (57). The application of high levels of phenols convert the semiquinone radicals by donating the electrons to molecular oxygen, forming superoxide anions (O^{2-}) and H_2O_2 which damages the membrane system (45).

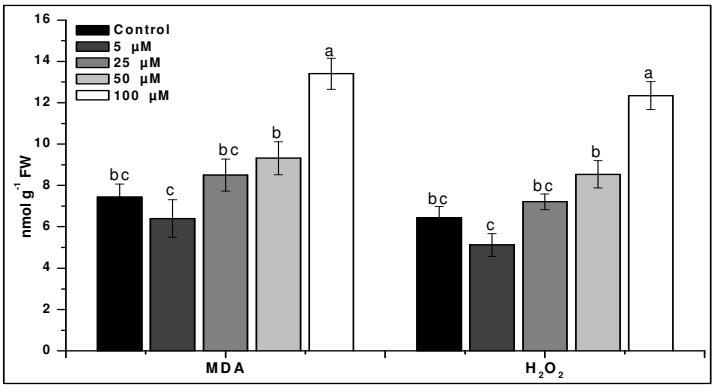

Figure 3. The effect of tricin on (a) MDA and (b) H_2O_2 levels of treated wild oat seedlings 10 d after sowing. Values are means of 5 replicates ± SE. Values with similar letters are not significantly different at p =0.05.

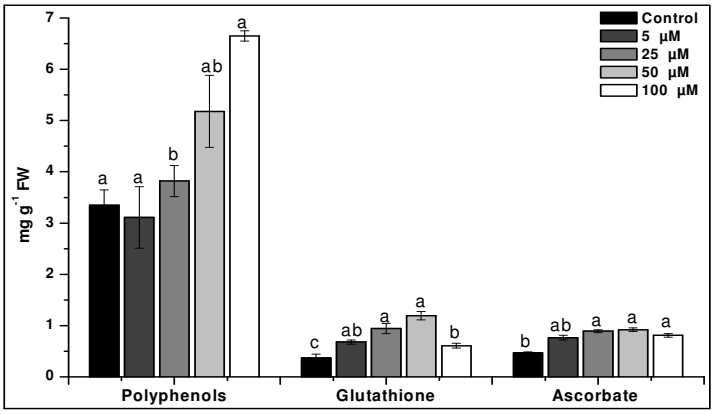

Figure 4. The effect of tricin on phenolic, glutathione and ascorbate contents of treated wild oat seedlings 10 d after sowing. Values are means of 5 replicates ± SE. Values with similar letters are not significantly different at p =0.05.

Antioxidant defence system

Antioxidant molecules participating in the cell protection against oxidative stress are polyphenols (29), ascorbate and glutathione (32,41). Under stress, plants accumulate these metabolites that act as non-enzymatic ROS-scavengers (56) and prevent the plant from damage. Tricin application stimulated the antioxidant metabolites (polyphenol, ascorbate and glutathione) of wild oat. Although the tricin increased the levels of antioxidant metabolites viz., polyphenol, (98.5%), glutathione (224%) and ascorbate (74.6%) (Fig. 4), but was not sufficient to prevent the plant damage by lipid peroxidation and H_2O_2 accumulation in wild oat shoots.

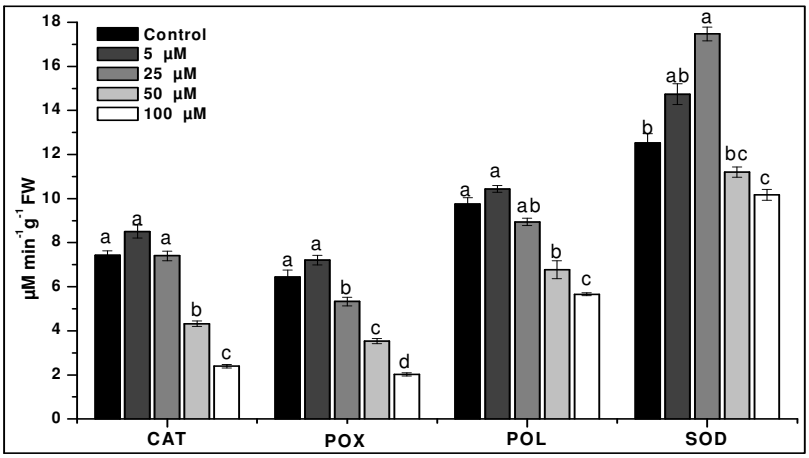

Figure 5. The effect of tricin on activities of catalase (CAT) (µM H_2O_2 min^{-1} g^{-1} FW), peroxidase (POX) (A420, µM purpurogallin min^{-1} g^{-1} FW), polyphenol oxidase (POL) (A420), µM purpurogallin min^{-1} g^{-1} FW and superoxide dismutase (SOD) (U g^{-1} FW) 10 d after sowing. Values are means of 5 replicates ± SE. Values with similar letters are not significantly different at p =0.05.

Treatment with higher tricin concentrations (25,50 and 100 µM) suppressed the activities of all measured anti-oxidant enzymes (CAT, PRX and POL) except SOD, which was slightly stimulated at 25 µM (Fig. 5). At 100 µM tricin there was maximum suppression in activities of CAT (67.7%), PRX (68.6%), POL (53.2%) and SOD (18.8%) with respect to control. The inhibitory effects of enzyme activities at higher concentrations were due to inhibition in their synthesis or by the inactivation and down regulation of certain defence enzymes (17). At high concentration, the bioactive compounds might directly inhibit the oxidizing enzymes in some way, leaving the plant vulnerable to oxidative damage (39,60).

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