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Bioassay-guided isolation of phytotoxins from three *Salvia* species

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ABSTRACT

This study aimed to test the phytotoxicity of plant parts of *Salvia miltiorrhiza* Bunge, *S. hispanica* L. and *S. columbariae* Benth. Roots and shoots were fractionated with hexane, ethyl acetate and ethanol and the fractions were tested on lettuce (*Lactuca sativa* L.) and creeping bentgrass (*Agrostis stolonifiera* L.). The hexane and ethyl acetate fractions of *S. miltiorrhiza* were sufficiently phytotoxic for further bioassay-guided fractionation. The abietane diterpenes tanshinone I and tanshinone IIA were the main phytotoxins found in the fractions. They inhibited growth of duckweed (*Lemna paucicostata* Hegelm. 6746) by 50 % at 113 and 140 µM concentrations, respectively. This level of phytotoxicity was similar to that of some commercial herbicides in this bioassay. Neither compound was toxic to mosquitoes (*Aedes aegypti* L.) at 125 ppm (ca. 0.435 mM). Although these compounds are red pigments reported to be weak photosensitizers, a cucumber (*Cucumis sativus* L.) cotyledon assay indicated that this is not their phytotoxic mode of action.

Key words: Allelochemical, bentgrass, bioassay guided isolation, cucumber, duckweed, lettuce, mosquito, permethrin, phytotoxins, *Salvia*, tanshinone.

INTRODUCTION

The genus *Salvia* includes around 900 species distributed throughout the world and is the largest genus in the economically and medicinally important plants of the Lamiaceae family. *Salvia* produces diverse diterpenoids, such as tanshinone IIA, salvicine and neotanshinlactone, many of which have significant bioactivities (25). The secondary compounds of *Salvia* species have been extensively studied for numerous biological activities (8,19,40). These studies focussed on radical scavenging molecules, as well as antimicrobial, antifungal and medicinal compounds. For example, the extensive review of Wu *et al*. (40) does not mention the research done on the phytotoxicity of some compounds isolated from *Salvia* species. Jassbi *et al*. (19) listed compounds from *Salvia* species that are phytotoxic. Both reviews catalogue compounds that have antifeedant and insecticidal effects.

Salvia miltiorrhiza Bunge (Red sage) is a traditional Chinese medicinal herb that is highly valued for its roots. The plant is deciduous perennial and 30 to- 60 cm tall. The species name *miltiorrhiza* means "red juice extracted from a root". It has been used to treat and prevent cardio-vascular disease, hyperlipidemia and cerebro-vascular disease. It has antioxidant, antimicrobial, antivirus, anticancer and anti-inflammatory activities (38).
Most of the allelochemical/phytotoxin work on *Salvia* spp. has focused on essential oils and their constituents. *S. leucophylla* was reported allelopathic many years ago (28,29,30). The

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volatile monoterpenes 1,8-cineole and camphor were identified as allelochemicals (29). β-Pinene was later added to the allelochemicals of *S. leucophylla* (31). *S. officianalis* has an array of phytotoxic terpenes, including 1-8-cineole, camphor, carvacrol, thymol, carvone and limonene (9). Mancinini *et al.* (26) found 1-8-cineole and camphor to be phytotoxic constituents of *S. hierosoloymitana* and *S. muilticualis* essential oils. The phytotoxicity of shoot exudates of 13-*Salvia* spp. were examined by Bisio *et al.* (5). Significant activity was found, but the responsible compounds were not isolated and identified. Bisio *et al.* (7) found several phytotoxic clerodane diterpenes from leaf surface exudates of *S. miniata.* In the only paper that we have found on bioassay-guided isolation of phytotoxic compounds from a *Salvia* species, Bisio *et al.* (6) reported several diterpene and flavonoid phytotoxins from *Salvia x jamensis.* Rial *et al.* (32) examined diterpenes and clerodanes from *S. gesneriflora, S. herbacea, S. polystachaya, S. shannoni, S. tiliaefolia and S. microphylla,* but found either no phytotoxicity or very weak phytotoxicity.

Pharmacological investigations have shown that the medicinally active constituents of *S. miltiorrhiza* are divided into two groups: water soluble phenolic acids and lipophilic diterpenoid tanshinones (22). However, the contents of these components in *S. miltiorrhiza* vary with collection location and time of harvest (45). According to Sun *et al.* (35), the major active constituents of this herb are tanshinones. Several tanshinone compounds have been isolated from *S. miltiorrhiza*, and most of them have shown biological activities. Some tanshinone compounds are used as reference standards in the quality control of *S. miltiorrhiza* and its products.

Although there are several reports of phytotoxins from *Salvia*, there are no reports of phytotoxins against higher plants from *S. miltiorrhiza*. Duke (14) relates that the finding of highly biologically active compounds used in medicine, can suggest that the compound might be a phytotoxin. However, there are studies about algicidal activity of *S. miltiorrhiza* roots against the bloom-forming cyanobacterium *Microcystis aeruginosa* (42). A significant inhibition of the algae at 800 mg L^{-1} for methanolic extracts was observed. The fraction of the ethyl acetate extract of *S. miltiorrhiza* was more efficient than others with the inhibitory rate (IR) of 91.3 % and 50 % effective concentration (EC_{50}) values at 98.9 mg L^{-1}, followed by chloroform extracts with an EC_{50} value of 111.5 mg L^{-1}.

Another study isolating compounds from the root of *S. miltiorrhiza*, found neo-przewaquinone A (43). It had algicide potential, with an EC_{50} value on *M. aeruginosa* of 4.68 mg L^{-1}. In addition, this compound showed relatively lower activity on *Chlorella pyrenoidosa* and *Scenedesmus obliquus* (EC_{50} values of 14.78 and 10.37 mg L^{-1}, respectively). However, Lürling and Oosterhout (24) tested root extracts of *S. miltiorrhiza* for suppression of the cyanobacterium *and* they had no significant effect on *M. aeruginosa* biomass. This difference could be due to different extraction procedures, as they used water extract. According to Duke (14,15), hydrophilic compounds may be less effective allelochemicals than lipophilic compounds because they can leach from the soil and are sometimes not taken up as lipophilic compounds.

Salvia hispanica L. (Chia) originated in Mexico and Guatemala, where its seeds have been used as human food for about 5500 years (37). It has also been used in a wide range of folk medicines, cosmetics and in religious rituals, and it has also been reported to have antioxidant, antimicrobial, antivirus, anticancer and anti-inflammatory activities (20). Aqueous extracts of its leaves were reported to inhibit the growth and development of wheat (*Triticum aestivum* L.) (13).

Salvia columbariae Benth. (Gold chia) grows in the western USA from California to Utah and south to northern Mexico. The seeds have been used as human food. Its root extracts contain tanshinones and similar compounds like cryptotanshinone that is a precursor for tanshinone IIA (1). We find no reports of allelopathy for phytotoxins associated with this species.

Considering the previous studies of the broad biological activities, including phytotoxicity of compounds from *Salvia* species, this study aimed to use bioassay-guided isolation for identification of the phytotoxic compounds in shoots and roots of *S. miltiorrhiza*, *S. hispanica* and *S. columbariae*, three species for which there is no literature identifying specific phytotoxins. Of these species, only *S. miltiorrhiza* was found to have sufficient phytotoxic compounds for bioassay-guided isolation, and tashinone I and tashinone IIA were the only compounds with significant phytotoxicity from *S. miltiorrhiza*.

MATERIAL AND METHODS

Plant Material and Extraction

Salvia miltiorrhiza, S. hispanica and *S. columbariae* seeds were bought from Horizon Herbs® (Williams, Oregon 97544, USA). Plants were grown in a greenhouse in Oxford, Mississippi, at 24 °C, in a 50/50 mixture of Metro-Mix 350 (a greenhouse growth medium containing vermiculite, sphagnum peat moss, bark ash, nutrients *and* dolomite limestone) (Sun Gro Horticulture, Agawam, MA 01001, USA) and soil conditioner (finely ground pine (*Pinus* spp.) bark from Sims Bark Co., Inc. (Tuscumbia, AL 35674, USA) in 7.3-L pots from May to July, 2015. Fresh roots and shoots from plants were dried in a lyophilizer, providing 499.5 g of roots and 178.6 g of shoot material from *S. miltiorrhiza and* 90.0 g of roots and 267.3 g of shoot material from *S. hispanica and* 4.8 g of roots and 85.3 g of shoot material from *S. columbariae*. After grinding, each plant part was soaked in solvents of increasing polarity. Briefly, plant material was soaked at room temperature in hexane, ethyl acetate and ethanol sequentially, for 24 h in each solvent followed by Buchner funnel filtration, rotary evaporation and nitrogen or lyophilization. The yields of root and shoot extractables from species were calculated based on mass (Table 1).

Gas Chromatography/Mass Spectrometry (GC/MS) Analysis

Samples were analyzed by GC/MS on an Agilent (Santa Clara, CA) 7890A GC system equipped with an Agilent 5975C inert XL MSD with triple axis detector and an Agilent 7693 autosampler. The Agilent GC/MS was equipped with a DB-5 column (30 m x 0.25 mm fused silica cap. column, film thickness of 0.25 µm) operated using the following conditions: injector temp., 240 °C; column temp., 60 to 240 °C at 3 °C/min, held at 240 °C for 5 min; carrier gas, He; MS mass range from 50 to 550 *m/z*; filament delay of 3.5 min; injection volume, 3 µL (split ratio 50:1).

Phytotoxicity-guided Fractionation of *S. miltiorrhiza*

Fractionation of the extracts was done with column chromatography using a Biotage, Inc. Horizon™ Pump (Charlottesville, VA, USA) equipped with a Horizon™ Flash Collector and fixed wavelength (254 nm) detector. Initially 630 mg and 655 mg of hexane and ethyl acetate extractable material, respectively, from the roots was separated on a Biotage 40+M column (normal phase 40–63 µm, 60 Å, 40 x 150 mm) running at 40 ml min-1 using a n-hexane:acetone step gradient beginning with 100:0 to 80:20 over 2,000 ml

followed by 80:20 to 50:50 over 800 ml and 50:50 to 0:100 over 400 ml. Fractions of 22 ml were collected and recombined, based on TLC similarities and UV chromatogram (254 and 280 nm) peak profiles, into 9-distinct fractions of 618.1 mg total from hexane extract and 10-distinct fractions of 357.5 mg total from ethyl acetate extract (Table 2) and submitted for bioassay. Fraction D from the hexane extract and fraction B from the ethyl acetate extract were identified as tanshinone IIA (Figure 1) using ^{1}H NMR, TLC and comparison of chemical shift data and Rf with an authentic standard (from Sigman-Aldrich). Fraction D from both extracts contained tanshinone IIA and fraction H from the hexane extract, and fraction E from the ethyl acetate extract contained tanshinone I (Figure 1) which was identified using ^{1}H NMR, TLC and comparison of chemical shift data and Rf with that from an authentic standard (from Sigma-Aldrich). Fractions E and G from the hexane extracts were selected for further investigation, based on activities in the phytotoxicity bioassays against lettuce and bentgrass (*Agrostis stolonifera*). Sub-fraction E-C was identified as tanshinone I.

Tanshinone I: ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.23 (d, J = 8.9 Hz), 8.25 (d, J = 8.6 Hz), 7.69 (d, J = 8.6 Hz), 7.54 – 7.48 (m, 1H), 7.34 (d, J = 7.0 Hz), 2.64 (s), 2.27 (s).
Tanshinone IIA: ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.59 (d, J = 8.2 Hz), 7.50 (d, J = 8.1 Hz), 7.20 – 7.16 (m), 3.16 (t), 2.27 – 2.17 (m), 1.75 (dd, J = 7.7, 4.8 Hz), 1.66 – 1.60 (m), 1.29 (s).

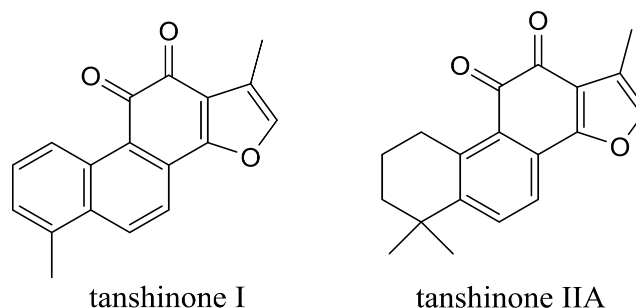

Figure 1. Structures of two phytotoxic compounds identified by bioassay-guided isolation from *Salvia miltiorrhiza*.

Bioassays with *Lactuca sativa* and *Agrostis stolonifera* without Soil

Silica gel column chromatographic fractionation of the hexane, ethyl acetate and ethanol extracts of the shoots and roots of *Salvia miltiorrhiza, S. hispanica* and *S. columbariae* were guided by lettuce (*L. sativa*) and bentgrass (*A. stolonifera*) bioassays according to Dayan *et al*. (10) to isolate phytotoxic fractions. A filter paper (Whatman #1) and 5 mg of *L. sativa* seeds or 10 mg of *A. stolonifera* seeds were placed in each well of a 24-well plate (Corning Inc., Corning, NY, USA). Test fractions were dissolved in acetone or water in ratio of 1 to 10 (test material to solvent), depending on the solubility of extractables, to obtain stock solutions, which were then tested by adding 20 µl stock solution to 180 µl of distilled deionized (DDI) water to give a test concentration of 1 mg/ml. All samples including extract free controls were adjusted to a final concentration of 10 % acetone. Plates were covered, sealed with parafilm and incubated at 24 °C in a growth

chamber (Conviron) at 173 µmol m^{-2} s^{-1} continuous photosynthetically active radiation (PAR). Phytotoxicity was qualitatively evaluated by visually comparing seed germination and seedling growth in each well with the extract-free 10 % acetone solution after 7 d. Phytotoxicity was estimated by using a rating scale of 0 to 5, where 0 = no effect and 5 = no germination of the seeds (Table 1).

Cellular Leakage Bioassay

Effects of tanshinone I and tanshinone IIA on cellular leakage of cucumber (*Cucumis sativa* L.) cotyledon discs was determined by the method of Dayan and Watson (12). Briefly, cucumber seedlings were grown in growth chamber with 16/8 light/dark cycle for 10 days. Twenty-five 4-mm cotyledon discs were placed on a 2 % sucrose/1 mM 2-(N-morpholino) ethane sulfonic acid buffer (MES, pH 6.5) containing different concentrations of tanshinone I or tanshinone IIA. Each plate contained 5 mL of buffer. Control tissues were exposed to the same amount of acetone as treated tissues but without the test compounds. The final concentration of acetone in the dishes was 1 % (v/v). Plates were incubated in dark for 16 h prior to exposure to high light intensity (1000 µmol m^{-2} s^{-1}) PAR in an incubator (Model E-30-B, Percival Scientific, Boone, IA 50036). Measurements were made using an electrical conductivity meter (Model 1056, Amber Science, Eugene, OR 97402) equipped with a model 858 Conductivity Macro Flow cell at the beginning of the dark incubation period, another measurement was made after 16 h (overnight), at which time the samples were placed in the light and a final measurement was made after 8 h of light exposure. Maximum conductivity was measured by boiling three samples of each treatment for 20 min. Concentrations causing 50 % inhibition of growth were determined with R Studio software (v.0.99.491).

Bioassays with duckweed *(Lemna paucicostata*)

Bioassays were done as previously described in detail (27). Briefly, *Lemna paucicostata* stocks were grown from a single colony consisting of a mother and two daughter fronds in a glass jar on modified Hoagland media containing 1,515 mg L^{-1} KNO_3, 680 mg L^{-1} KH_2PO_4, 492 mg L^{-1} $MgSO_4$ •$7H_2O$, 20 mg L^{-1} Na_2CO_3, 1,180 mg L^{-1} Ca $(NO_3)_2$ •$4H_2O$, 0.5 mg L^{-1} $MnCl_2$, 0.025 mg L^{-1} $CoCl_2$, 0.025 mg L^{-1} $CuSO_4$ •$5H_2O$ and 18.355 mg L^{-1} Fe-EDTA. The media was adjusted to pH 5.5 with 1 M NaOH and filtered through a 0.2 µm filter. Each well of a nonpyrogenic polystyrene sterile six-well plate (CoStar 3506, Corning Inc., Corning, NY, USA) was filled with 4,950 µl of the Hoagland media mixed with 50 µl of acetone or 50 µl of acetone with the appropriate concentration of tanshinone I and tanshinone IIA. Independent experiments demonstrated that there was no effect of 1 % acetone on growth of the plants. Two three-frond colonies from 4-to 5-d-old stock cultures were placed in each well. Total frond area per well was recorded by the image analysis system, Scanalyzer (LemnaTec, Würselen, Germany) at 4 and 7 d. Percent increase at 4 to 7 d was determined relative to baseline area at day 0.

Mosquito Larval Bioassays.

Bioassays were conducted to test tanshinone I and tanshinone IIA for their larvicidal activity against *Aedes aegypti* by using the bioassay system described by Ali *et al*. (2). Eggs were hatched and larvae held overnight in the hatching cup in a temperature-controlled room. Five 1-d-old *Ae. aegypti* larvae were added in a droplet of water to each well of 24-well plates (BD Labware, Franklin Lakes, NJ) by use of a disposable 22.5 cm Pasteur

pipette. Fifty microliters of larval diet (2 % slurry of 3:2 beef liver powder (Now Foods, Bloomingdale, Illinois) and Brewer's yeast (Lewis Laboratories Ltd., Westport, CT) was added to each well by using a Finnpipette stepper (Thermo Fisher, Vantaa, Finland). All samples tested were diluted in dimethyl sulfoxide (DMSO). Eleven microliters of the test chemical was added to the labelled wells, while 11 µL of DMSO was added to control treatments. Permethrin (95.7 %) (Chem Service, Inc. West Chester, PA) was used as a positive control. After the treatment application, the plates were swirled in clock-wise and counter clockwise motions, front and back and side to side five times to ensure even mixing of the chemicals. Larval mortality was recorded 24-h post treatment. Larvae that showed no movement in the well after manual disturbance of water were recorded as dead.

RESULTS AND DISCUSSION

There was no strong phytotoxicity of any of the fractions from roots or shoots of either *S. hispanica* and *S. columbariae* using both the lettuce and bentgrass bioassays (Table 1). However, the ethyl acetate extracts of both roots and shoots of *S. miltiorrhiza* were more phytotoxic, as was the hexane extract of the roots (Table 1). The hexane and ethyl acetate root extracts were further fractionated into 10-fractions (Table 2) and the highest activity was found in root hexane fractions E and G, which were further fractionated (Table 3). Fraction D from the hexane extract and fractions B and D from the ethyl acetate extract were identified as tanshinone IIA (Figure 1) using ^{1}H NMR, TLC and comparison of chemical shift data and Rf with an authentic standard. Fraction H from the hexane extract and fraction E from the ethyl acetate extract contained tanshinone I (Figure 1), also identified using ^{1}H NMR, TLC and comparison of chemical shift data and Rf with an authentic standard.

Table 1. Amount of material recovered in each extract and its phytotoxicity*

Salvia species	Part (g)	Extraction Solvent	Extract Weight (g)	Phytotoxicity (1mg/ml)	
				Lettuce	Bent grass
S. miltiorrhiza	Root	Hexane	2.575	4	3
	(499.54)	Ethyl Acetate	3.842	4	3
		Ethanol	23.51	0	2
	Shoot	Hexane	4.315	0	0
	(178.61)	Ethyl Acetate	3.624	3	1
		Ethanol	9.353	0	0
S. hispanica	Root	Hexane	0.392	1	1
	(90.01)	Ethyl Acetate	0.382	1	1
		Ethanol	1.314	0	0
	Shoot	Hexane	3.695	0	2
	(267.27)	Ethyl Acetate	4.336	3	2
		Ethanol	8.667	1	1
S. columbariae	Root	Hexane	0.028	0	1
	(4.76)	Ethyl Acetate	0.042	0	0
		Ethanol	0.097	0	0
	Shoot	Hexane	5.383	0	1
	(85.27)	Ethyl Acetate	1.808	2	3
		Ethanol	2.279	0	0

* Values denote toxicity at 1.0 mg/ml to lettuce and bentgrass. 0: no effect, 5: no germination.

Fractions E and G from the hexane extracts were selected for further investigation, based on activities in the phytotoxicity bioassays against lettuce (*L. sativa*) and bentgrass (*A. stolonifera*).

Table 2. Amount of material recovered in each fraction and its phytotoxicity* from root of *Salvia miltiorrhiza*

Extracts/Fractions		A	B	C	D	E	F	G	H	I	J
Hexane (630 mg)	Weight (mg)	7.7	57.5	41.1	63.1	266.5	55.6	59.8	19.1	47.7	-
	Lettuce	1	0	3	3	4	3	4	4	3	-
	Bentgrass	0	3	4	3	3	4	4	4	4	-
Ethyl acetate (655 mg)	Weight (mg)	1.9	15.9	21.5	16.7	26.8	77.6	58.9	77.6	29.7	30.9
	Lettuce	0	1	1	2	4	0	2	2	0	3
	Bentgrass	0	4	3	3	4	0	0	3	0	3

* Values denote toxicity at 1.0 mg/ml to lettuce and bentgrass. 0: no effect, 5: no germination

Table 3. Amount of material recovered in each sub-fraction and its phytotoxicity* from hexane extracts of root of *Salvia miltiorrhiza*

Fraction/Sub-fractions		A	B	C	D	E	F
Hexane Fraction E (227 mg)	Weight (mg)	7.1	4.9	15.7	15.6	6.0	45.0
	Lettuce	0	0	2	0	0	0
	Bentgrass	0	0	1	0	0	0
Hexane Fraction G (151 mg)	Weight (mg)	12.5	28.4	74.0	18.7	12.8	-
	Lettuce	0	3	3	0	0	-
	Bentgrass	0	3	3	0	0	-

* Values denote toxicity at 1.0 mg/ml to lettuce and bentgrass. 0: no effect, 5: no germination.

Even though *S. columariae* is reported to contain tanshinone IIA, its content was found much lower than that of *S. miltiorrhiza* (1). Thus, the fractions of *S. columariae* in which tanshinone IIA would be expected probably had too little of the compound to provide a sufficient level of phytotoxicity in the bioassay that we used. Also, the concentration of this compound in the *S. columariae* that we grew may have been limited by the growing conditions and/or the genetics of thebiotype. Pure tanshinone I and tanshinone IIA were then assayed for phytotoxicity against duckweed (Figure 2). Tanshinone I and tanshinone IIA inhibited growth 50 % at 113 and 140 µM, respectively, 7 days after exposure. This level of activity was similar to commercial herbicides clomazone and EPTC (*S*-ethyl dipropylcarbamothioate) after 7 days of exposure in the same bioassay (27). Both compounds stimulated plant growth at a concentration just below the lowest inhibitory concentration. The stimulation of a growth parameter by a subtoxic concentration of a toxin is termed hormesis. The hormetic effects observed here were similar to the hormetic effects of many herbicides and allelochemicals on plant growth (4,16).

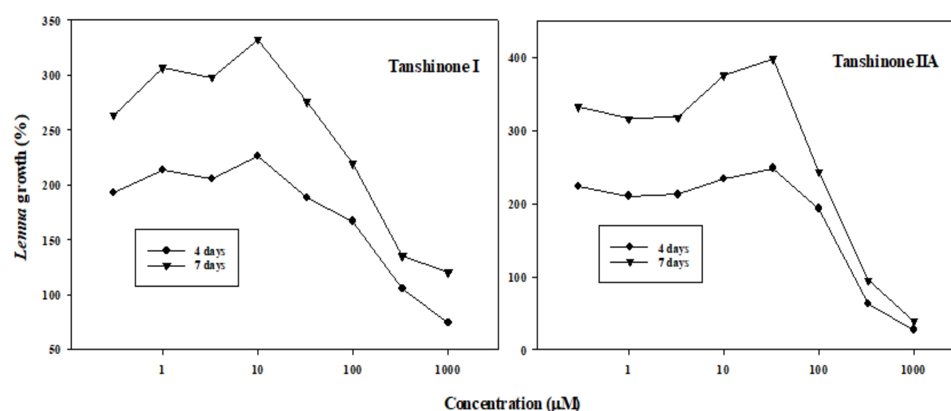

Figure 2. Dose/response curve for tanshinone I and tanshinone IIA against duckweed growth at 4 and 7 days after exposure. The first data point in each plot is the control value.

Tanshinone IIA and tanshinone I are weak photosensitizing, red pigments (e.g., 21,44). Therefore, we used the cucumber cotyledon disc bioassay (12) that is sensitive in determination if a phytotoxin is a photosensitizer or causes accumulation of endogenous photosensitizing pigments. There was no marked electrolyte leakage caused by either compound at up to 330 µM concentration in darkness or light (Figure 3).

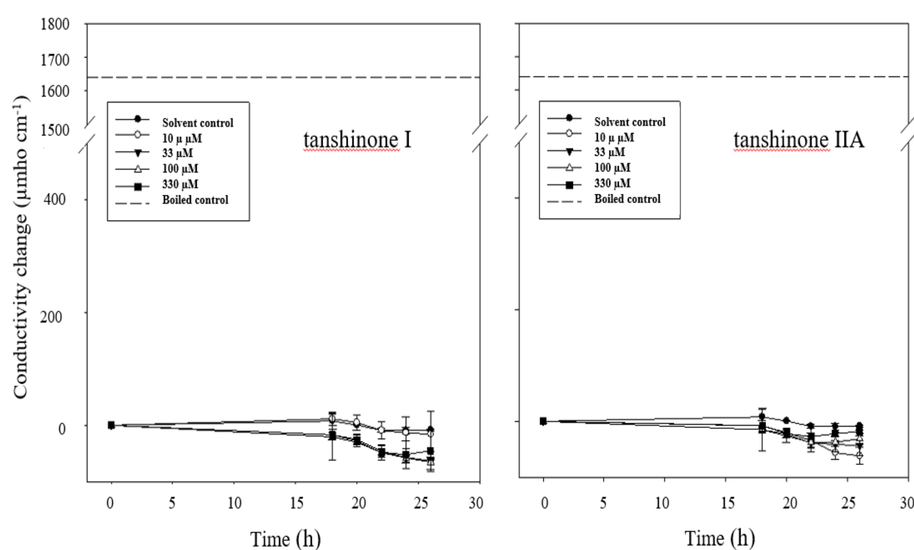

Figure 3. Effects of different concentrations of tanshinone I and tanshinone IIA on cellular leakage of cucumber cotyledon discs during an 18 h dark period, followed by an 8 h light period starting at the arrow. The dotted line lines are maximal possible leakage with 100 % of cell disruption.

The phytotoxins previously reported from *Salvia* species did not include tashinones at the time of Jassbi *et al*. (19) review. We were surprised that there were no reports of these compound's phytotoxicity in literature, especially because several *Salvia* species are known to be allelopathic and the tanshinones are diterpene quinones and several well-known allelochemicals such as juglone (39) and sorgoleone (11) are quinones. Quinones are usually biologically active.

Even though these two compounds are not highly phytotoxic, the amount found in the roots of *S. miltiorrhiza* could compensate for their relatively weak activity compared to some other allelochemicals found in other plant species at lower concentrations. The amount of tanshinones in *S. miltiorrhiza* roots can reach 12 mg/g of dry weight (33). The amount of phytotoxin produced by a plant is a pivotal aspect of allelopathy (17,18). For example, through bioassay-guided isolation of allelochemicals, the allelopathy of *Carduus nutans* and *C. acanthoides* was found to be due to the high levels of weakly phytotoxic aplotaxene in the roots (34).

How tanshinones act as phytotoxins is unknown. There is considerable literature on how chemicals affect tanshinone production in *S. miltiorrhiza* (e.g., 41), but nothing on the effects of tanshinone on plant physiology or biochemistry of other plant species. At least some of the activity of tanshinones in animals is due to inhibition of tyrosine kinases, important enzymes in signalling (36). Plants apparently also have tyrosine kinases (3,23), so effects on plant tyrosine kinases could account for the phytotoxicity of tanshinones.

Some *Salvia* species are reported to have insect-active compounds, but the extensive review of Jassbi *et al*. (19) does not mention the tanshinones as having such activity, nor do they mention that they do not have such activity. The effects of two compounds were tested for larvacidal activity on *A. aegypti* at different concentrations. The highest concentration of the tanshinones tested (125 ppm for both; that is 0.425 mM for tanshinone IIA and 0.453 mM for tanshinone I) had no effects on the larvae after 24 and 48 h of exposure. Permethrin produced 100% mortality at 125 ppm by 24 h exposure.

CONCLUSIONS

The only significant phytotoxins found in our survey of three *Salvia* species were tanshinone I and tanshonone IIA in *S. miltiorrhiza*. These red pigments that can act as photosensitizing agents do not act as phytotoxins through this mechanism. The function of these compounds in contributing to the survival of this species in nature has yet to be determined.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest : The Authors declare no conflict of Interest.

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