

EFFECT OF SALICYLLIC ACID FOLIAR APPLICATION ON PHYTOCHEMICAL COMPOSITION, ANTIOXIDANT AND ANTIMICROBIAL ACTIVITY OF *SILYBUM MARIANUM*

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ABSTRACT

Silybum marianum (L.), is an important herbal medicine. Silymarin, the active component obtained from its edible seeds, is known for its antioxidant and antimicrobial activity. This research was aimed to study the effect of foliar application of salicylic acid (SA) at four concentrations of 1250, 2500, 5000 and 10000 $\mu\text{mol l}^{-1}$ on the improvement of phytochemical composition, antioxidant and antimicrobial activity. Significant increase was found in the content of silibinin, silybin A and silybin B in plants treated with increasing concentrations of salicylic acid. The antioxidant activity was improved with increasing the SA concentration reaching the highest amount under 5000 $\mu\text{mol l}^{-1}$ SA treatment ($p < 0.05$). Irrespective to the bacterial strain, an increasing pattern in the amount of antibacterial activity was found by increasing SA concentration. The study suggest that treatments with medium doses of SA could be a promising way to improve the health beneficial flavonolignans compounds of *Silybum marianum* resulting in a higher antioxidant and antimicrobial activity. Nevertheless, it should be considered that the responses to SA are highly concentration dependent and application of higher concentrations can have an adverse effect by triggering a hyper sensitive cell death pathway.

Key words: antioxidant, SA. concentration, growth regulator, milk thistle, silymarin

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تأثير الرش بحامض السالسليك في التركيب الكيميائي والفعالية المايكروبية زفعالية مضادات الاكسدة لنبات الخرفيش

SILYBUM MARIANUM

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لمستخلص

تهدف الدراسة التحري عن تأثير رش حامض السالسليك باربعة تراكيز (1250 , 2500 , 5000 , مايكرومول لتر⁻¹) في تحسين التركيب الكيميائي للنبات والفعالية ضد ميكروبية ومضادات الاكسدة . أظهرت النتائج حصول زيادة معنوية في محتوى النبات من سليبينين , سليفباين أ و سليباين ب للنباتات المعاملة بتراكيز من السالسليك و لغاية التركيز 5000 مايكرومول لتر⁻¹ . كما سجلت فعالية ضد مايكروبية بزيادة تراكيز السالسليك . أوضحت النتائج ان المعاملة بجرعة متوسطة ينتج عنه ينتج عنه زيادة في محتوى النبات من مضادات الاكسدة و الفعالية ضد مايكروبية وعليه الاستجابة لحامض السالسليك تعتمد على التراكيز المستعمل . في حالة استعمال تراكيز عالية سبب موت بعض الخلايا .

كلمات مفتاحية: مايكروب، سليبينين، سليفباين أ، سليفباين ب

INTRODUCTION

Silybum marianum (L.), also known as milk thistle, is an important herbal medicine. This plant is native to the Mediterranean basin and is widespread in northwest and south of Iran (23). Historically, milk thistle was grown as a food source. The roots can be eaten; flower receptacle eaten like artichokes; the leaves eaten as a spinach substitute; the stalks eaten like asparagus (27). Seeds of milk thistle have been used from centuries for the treatment of liver diseases (hepatitis, cyrosis and icterus), blood cholesterol and containment of cancer (15, 22). The active component obtained from its edible seeds is known as silymarin. It contains 65-85% flavonolignans like silychristin, isosilychristin, silydianin, silybin A and B, isosilybin A and B and also 20-35% fatty acids, flavonoids and other polyphenolics (7). Given the important pharmacological effects associated with silymarin, many studies have been conducted on the biological activities of these compounds. The liver-protecting abilities of silymarin are due to the antioxidant and free radical scavenging properties. Silymarin has the ability to scavenge free radicals through increasing the production of glutathione in hepatocytes and the activity of superoxide dismutase in erythrocytes (25). Studies have reported the synergistic activity of silibinin when combined with ampicillin and gentamicin against bacteria that attack the oral cavity (16). However, there are few works that evaluated the antimicrobial capacity of silymarin and silibinin, demonstrating the need to extend the study of their therapeutic use in this regard (6). Given the importance of medicinal plants and their biological compounds, the management practices which help to improve the quality of medicinal plants has been in the center of attention. The induction of signaling pathways through the external application of variety of compounds can promote the biosynthesis of phytochemicals. One of the compound widely used as a signaling molecule to improve the biosynthesis of phytochemicals is salicylic acid (SA) (16, 18). SA is as an endogenous plant growth regulator which controls a large variety of physiological processes (8). It is a regulatory signal that mediates plant's defense mechanisms and their response to abiotic

stresses (19). Exogenous application of SA is shown to induce many physiological changes including prevention of ethylene production (14); increases in plant growth factor (20) and antioxidant activity (3). This study, was aimed to investigate the effect of foliar application of SA on the (1) the production of major bioactive components of silymarin including silibinin, silybin A and silybin B (2) the amount of antioxidant activity and (3) the degree of antibacterial activity.

MATERIALS AND METHODS

Growing plants and applying treatments

The experiments were conducted in the research center of Islamic Azad University Miyaneh Branch, Miyaneh, Iran in 2017. The seeds were obtained from Medicinal Plants Research Center (Ardabil) and grown in small pots filled with vermiculite by 2 kg soil. When the seedling were 15–20 cm high and had 2 real leaves, they were transplanted into bigger pots by 4 kg soils. The pots were irrigated every ten days according to needs of plants. The treatments started 30 days after transferring to the main pots. Treatments were established in a completely randomized experimental design using 15 plants per treatment with three replicates. Treatments consisted of Salicylic acid (SA-2 hydroxybenzoic acid, Merck Millipore Corporation, Germany) foliar application at four concentrations of 1250, 2500, 5000 and 10000 $\mu\text{mol l}^{-1}$. The solutions were applied to the plants every 10 days by spraying using a hand-held sprayer during the whole cultivation period (from vegetative to reproductive phase). In order to avoid interferences with different solution levels, the same amount of distilled water was sprayed to the control plants at a given time.

Extraction procedure

Silybum marianum seeds were grounded into fine powder using an electric grinder. In order to extract silymarin, five gram of finely powdered samples were weighed and extracted with 250 ml ethanol for 8 h in a Soxhlet apparatus (Sigma-Aldrich). The extract was allowed to evaporate at room temperature for a period of time till it reached the one third volume of the original extract. Then it was preserved at 4°C in vials (1).

Quantification of compounds by HPLC

The chromatographic analysis was carried out using a Knauer K2600A liquid chromatograph (Germany) equipped with a C18 column (150 × 4.6 mm) packed with 5 μm diameter particles. The mobile phase was a mixture of 85% phosphoric acid, methanol, and water (1:46:64, v v⁻¹). 10 micro liter of standards and the samples was injected into the HPLC system at 40 °C and UV detection. The flow rate was 1ml min⁻¹ and the wavelength was 288 nm. The identification of silybin (A and B) and silibinin was performed by comparing their retention time and UV absorption spectrum with those of the commercial standards (purity ≥98% grade HPLC, Sigma Aldrich, Germany) (11).

Antioxidant activity

The antioxidant activity was analyzed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. The reaction of DPPH was spectrophotometrically measured for 1800 s at 517 nm on a CamSpec M501 spectrophotometer. Different volumes of extract (150-350 μl) were added to 3 ml of absolute methanol, The diluted extracts were then mixed with 1 ml DPPH ethanolic solution in 96% ethanol, the decrease of the absorbance at 517 nm was measured after 30 minutes of storage at room temperature in the dark. The same procedure was applied for the hydroxytoluene butylate (BHT) as a positive control. The antiradical activity was expressed as IC₅₀ (μg ml⁻¹), the concentration of the extracts which caused 50% inhibitory activity. A lower IC₅₀ value corresponds to a higher antioxidant activity of extracts. The ability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = (A_0 - A_1)/A_0 \times 100 \quad (1)$$

where A₀ is the absorbance of the control at 30 min and A₁ is the absorbance of the sample at 30 min (2).

Antibacterial activity

Antibacterial activity of *Silybum marianum* extracts was determined against eight bacterial strains, *Staphylococcus aureus* (PTCC 1431), *Bacillus cereus* (PTCC 1015), *Salmonella enterica* (PTCC 1787), *Proteus mirabilis* (PTCC 1776), *Klebsiella pneumonia* (PTCC 1053), *Shigella dysenteria* (PTCC 1188), *Pseudomonas aeruginosa* (PTCC 1074) and

Escherichia coli (PTCC 1399) using the disc diffusion method. A single colony of each bacterial strain was inoculated from a streak plate into 10 ml Mueller Hinton Broth (MHB) medium containing tubes. These tubes were incubated at 37 °C and a speed of 200 rpm for 16-18 hours as a primary culture. 100 μl of primary culture was inoculated into 10 ml MHB media. The tubes were incubated at 37 °C and a speed of 200 rpm till the absorbance reached 0.3 at 600 nm. Then 25 ml agar was poured into the plates slowly, left to solidify and put in the incubator for 24 hours to see for any signs of growth. The next day the plates were clear and ready for inoculation with the bacteria. After inoculating with the bacteria, a sterile 5 mm paper disc was soaked in the crude extract of *Silybum marianum* and then placed over the surface of the inoculated nutrient agar in antibacterial assay. All plates were incubated at 37°C overnight. After incubation, the petri dishes were observed for zone of inhibition. The diameter of zone was measured for recording the clear zone compared with the Di methyl sulfoxide (DMSO) as a control. Experiments were performed in triplicate and mean inhibitory zone was calculated. The standard antibiotics, ampicillin and gentamicin, were used for comparison with the *Silybum marianum* extracts (4). The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of extracts was determined using the microdilution assay. A volume of 1 ml MHB medium was added to each tube and 1 ml of the extract was used to do a twofold serial dilution giving concentrations of 5 to 0.00781 mg ml⁻¹. Next, 100 μl of the bacterial suspension was added to all tubes except the negative control or blank. The negative control contained 1 ml of MHB medium and 1 ml of the extract. Meanwhile, the positive control contained the bacterial suspension MHB, medium and DMSO. The tubes were placed in an incubator for 24 h at 37 °C. MIC was defined as the lowest concentration at which no bacterial growth (turbidity) was observed. Next, 100 μl of tubes with no sign of bacterial growth was transferred and evenly spread on the ager plates. Following 24 h of incubation at 37 °C, the plates were checked for bacterial growth.

MBC was defined as the lowest concentration of antimicrobial agent that kills of bacteria (4).

Statistical analysis

Each experiment was performed three times replicates and the results were normalized by calculation of mean values and the standard deviation. Statistical analyses were performed using GraphPad Prism, version 5.02. Differences between treatments were examined using ANOVA (two-way analysis of variance). The means were compared by Bonferroni post test and considered statistically significant when $P < 0.05$ (GraphPad Software, Inc., San Diego, CA).

Content of flavonolignans

Table 1. Silibinin, Silybin A and Silybin B content in plants treated with different salicylic acid concentrations.

Treatment*	g Silybin A/100 g DW	g Silybin B/100 g DW	g Silibinin/100 g DW
No spraying	1.506 c ± 0.006	1.520 c ± 0.005	3.026 c ± 0.012
Water spraying	1.506 c ± 0.006	1.533 c ± 0.003	3.040 c ± 0.010
1250 SA ($\mu\text{mol l}^{-1}$)	1.590 b ± 0.005	1.600 b ± 0.015	3.190 b ± 0.020
2500 SA ($\mu\text{mol l}^{-1}$)	1.593 b ± 0.008	1.620 b ± 0.005	3.223 b ± 0.014
5000 SA ($\mu\text{mol l}^{-1}$)	1.633 a ± 0.008	1.653 a ± 0.008	3.286 a ± 0.016
10000 SA ($\mu\text{mol l}^{-1}$)	1.603 b ± 0.008	1.620 b ± 0.005	3.213 b ± 0.013

*Values with different letters at each column are statistically different ($P < 0.05$).

DW: Dry Weight

RESULTS AND DISCUSSION

Antioxidant activity: With the increase in the concentration of SA, the antioxidant activity was improved (Table 2).

The highest amount of antioxidant activity (indicated by the lowest amount of IC₅₀) was

Table 2. Antioxidant activity in plants treated with different salicylic acid concentrations and control

Treatment	DPPH radical scavenging activity (IC ₅₀ ($\mu\text{g ml}^{-1}$))
No spraying	55.66 c ± 0.577
Water spraying	55.66 c ± 0.577
1250 SA ($\mu\text{mol l}^{-1}$)	53.00 b ± 0.577
2500 SA ($\mu\text{mol l}^{-1}$)	51.33 b ± 0.666
5000 SA ($\mu\text{mol l}^{-1}$)	48.00 a ± 1.201
10000 SA ($\mu\text{mol l}^{-1}$)	52.33 b ± 0.881

Antibacterial Activity

Antibacterial activity was mainly found against Gram-positive bacteria. While the three of the Gram-negative bacteria were not observed in the inhibition zone, they which were mentioned in the Table 3 and 4. Irrespective to the bacterial strain, an

HPLC analysis of silymarin extract revealed a larger concentration of silibinin compared to silybin A and silybin B. Significant increase was found in the content of silibinin, silybin A and silybin B in plants treated with increasing concentrations of salicylic acid. The highest effect was found in plants treated with 5000 $\mu\text{mol l}^{-1}$ SA, while higher concentrations can have an adverse effect by triggering a hypersensitive cell death pathway (10). In this study also treatment with higher SA concentration (10000 $\mu\text{mol l}^{-1}$) resulted in a decrease in silibinin, silybin A and silybin B content (Table 1).

found under 5000 $\mu\text{mol l}^{-1}$ SA treatment. Further increase in the SA concentration had an adverse effect and resulted in a decrease in antioxidant activity under 10000 $\mu\text{mol l}^{-1}$ SA treatment.

increasing pattern in the amount of antibacterial activity was found by increasing SA concentration. The zone of inhibition for all listed bacteria increased with the increasing concentrations of SA and reached its peak in plant treated with 5000 $\mu\text{mol l}^{-1}$ SA (Table 3).

Table 3. Inhibition zone diameter of pathogenic strains in plants treated with different salicylic acid concentrations and control

Treatment	Inhibition zone diameter (mm)				
	<i>Staphylococcus aureus</i>	<i>Bacillus cereus</i>	<i>Salmonella enterica</i>	<i>Proteus mirabilis</i>	<i>Escherichia coli</i>
No spraying	8.833 ± 0.440	8.500 ± 0.500	8.366 ± 0.472	8.400 ± 0.360	8.033 ± 0.251
Water spraying	8.900 ± 0.493	8.500 ± 0.500	8.533 ± 0.321	8.466 ± 0.450	8.133 ± 0.152
1250 SA (µmol l ⁻¹)	13.666 ± 0.3333	13.500 ± 0.500	13.766 ± 0.251	14.400 ± 0.450	13.266 ± 0.251
2500 SA (µmol l ⁻¹)	15.700 ± 0.288	15.900 ± 0.500	15.500 ± 0.529	14.433 ± 0.378	13.750 ± 0.321
5000 SA (µmol l ⁻¹)	17.500 ± 0.288	17.466 ± 0.953	17.266 ± 0.458	16.400 ± 0.360	15.233 ± 0.100
10000 SA (µmol l ⁻¹)	15.500 ± 0.288	15.500 ± 0.450	15.400 ± 0.461	14.433 ± 0.360	13.366 ± 0.251
DMSO	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
Methanol	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00	0 ± 0.00
Ampicillin	30.333 ± 0.333	28.000 ± 1.000	24.466 ± 0.450	25.400 ± 0.360	25.500 ± 0.500
Gentamicin	32.333 ± 0.333	28.833 ± 0.763	25.533 ± 0.503	27.566 ± 0.513	26.733 ± 0.642

At this concentration (5000 µmol l⁻¹ SA), the inhibition zone was almost two times higher than the control. On the other hand, the MIC

and MBC decreased with increasing SA concentration indicating the improvement of antibacterial activity (Table 4).

Table 4. MIC/MBC of pathogenic strains in plants treated with different salicylic acid concentrations and control

MIC/MBC (mgml ⁻¹) Treatment	<i>Staphylococcus aureus</i>		<i>Bacillus cereus</i>		<i>Salmonella enterica</i>		<i>Proteus mirabilis</i>		<i>Escherichia coli</i>	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
No spraying	1.25	2.50	2.50	5.00	2.50	5.00	2.50	5.00	5.00	-
Water spraying	1.25	2.50	2.50	5.00	2.50	5.00	2.50	5.00	5.00	-
1250 SA (µmol l ⁻¹)	0.312	1.25	1.25	2.50	1.25	2.50	1.25	2.50	1.25	5.00
2500 SA (µmol l ⁻¹)	0.312	1.25	1.25	2.50	1.25	2.50	1.25	2.50	1.25	5.00
5000 SA (µmol l ⁻¹)	0.156	0.625	0.625	1.25	0.625	1.25	0.625	2.50	1.25	5.00
10000 SA (µmol l ⁻¹)	0.312	1.25	1.25	2.50	1.25	2.50	1.25	5.00	2.50	5.00

Among the studied bacteria, *Staphylococcus aureus* was the most sensitive to SA application, indicated by a decrease in MIC from 1.25 in control to 0.156 under 5000 µmol l⁻¹ SA treatment. Further increase of SA concentration into 10000 µmol l⁻¹ resulted in a decrease in the zone of inhibition and an increase in MIC and MBC in all studied strains. This indicates the negative effect of higher SA concentration on the antibacterial activity. The antimicrobial effects of positive control (gentamicin and ampicillin) on Gram-positive bacteria were higher than other bacteria with a mean zone of inhibition of 30 mm in *Staphylococcus aureus*. The negative control (methanol and DMSO) were ineffective in all strains. This study of silymarin extracts indicated silibinin as the major components, followed by silybin A and B. The higher concentration of silibinin has

been already reported in silymarin extracts (24). We revealed an increasing pattern of silymarin flavonolignans (silybin A, silybin B and silibinin) in response to the foliar SA application reaching the highest concentration at a dose of 5000 µmol l⁻¹. The effect of exogenous SA applications on the accumulation of flavonoids has been shown in several plant species (26). SA application in cell suspensions can induce biochemical stress resulting in an increase in phytochemical compounds (10). It has also been found that SA induces the production of hydrogen peroxide (H₂O₂), which stimulates a greater activity of phenylalanine ammonium lyase, responsible for the synthesis of phenolic compounds (12). An increasing pattern in antioxidant activity of silymarin extracts was also observed with increasing SA concentration. Regular applications of salicylic

acid at different stages of plant growth and fruit development can induce an increase in the antioxidant activity (21). Such influence of SA could be related to the accumulation of phenolic and flavonoids compound which are proved to have antioxidant activity. On the other hand, SA application can have a direct physiological effect on the activity of antioxidant enzymes (13). The effect of seed hormonal priming using SA on the activity of antioxidant enzymes has been recently studied in milk thistle seeds. The increasing concentrations of SA was shown to induce the activity of catalase, peroxidase and polyphenol oxidase (17). The antimicrobial activity of silymarin extracts against a wide range of microorganisms was previously reported (15). Findings have pointed to a synergistic drug-modifying effect when silymarin and silibinin were combined with antibiotics, especially aminoglycosides, against the different bacterial strains evaluated. This study indicated a significant increase in the antimicrobial activity of silymarin extracts with increasing SA concentrations. Such effect could be related to the accumulation of flavonolignans upon SA application. Previous studies have demonstrated a significant inhibitory effect of flavonoids on DNA topoisomerase activity by the formation of complexes that alter enzyme binding (28). The inhibition of this enzyme can therefore interfere with the bacterial synthetic processes (5). This study suggest that treatments with SA could be a promising way to improve the health beneficial flavonolignans compounds of *Silybum marianum* resulting in a higher antioxidant and antimicrobial activity. Nevertheless, it should be considered that the responses to SA are highly concentration dependent. It is therefore, advisable to use medium doses of SA (up to 5000 $\mu\text{mol l}^{-1}$).

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