Journal of Medicinal Plants and By-products (2020) 1: 7-16

Original Article

Green Tea (*Camellia sinensis*) Extract Induces Systemic Acquired Resistance against Witches' Broom Diseases of *Citrus aurantifolia*

Saeed Mollaei[1*] and Zohreh Ghanavi[2]

[1]*Phytochemical Laboratory, Department of Chemistry, Faculty of Sciences, Azarbaijan Shahid Madani University, Tabriz, Iran*
[2]*Institute of Standard and Industrial Research of Iran, Tehran, Iran*

Article History: Received: 14 April 2019/Accepted in revised form: 25 August 2019

Abstract

Witches' broom disease of *Citrus aurantifolia* (Christm.) Swingle, caused by '*Candidatus* Phytoplasma *aurantifolia'*, is a big threat to *Citrus aurantifolia* production in South of Iran. Accumulation of defense-related enzymes is defense reactions against pathogens in infected plant tissues. Herein, the effect of ethyl acetate extract of *Camellia sinensis* (L.) Kuntze was investigated on induced resistance against phytoplasma in the leaves of *Citrus aurantifolia*. The leaves were pretreated with different concentration of the extract, and then study on the defense enzymes and phenolic compounds was done. The results indicated that the pre-treatment of the leaves with low concentration of *Camellia sinensis* extract induced resistance through the accumulation of various phenolic compounds and phytoalexins, as well as the activation of peroxidases (POD), catalase (CAT) and key enzymes in phenylpropanoid pathways i.e., phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), and stilbene synthase (STS). Thus, applying low concentration of *Camellia sinensis* extract induces resistance against disease and can be a novel strategy in plant disease management.

Keywords: *Camellia sinensis*, *Citrus aurantifolia*, Phenolic compounds, Resistance enzymes.

Introduction

The devastating effect of *Candidatus* Phytoplasma *aurantifolia*, the causal agent of witches' broom disease of *Citrus aurantifolia* (Christm.) Swingle ,resulted in economic loss of *Citrus aurantifolia* trees in United Arab Emirates, Oman and Iran [1, 2]. The first symptoms of this disease in the field are one or more witches' brooms of thin proliferating twigs that have small, pale leaves. Additional witches' brooms appear as the disease progresses and extensive die-back occurs. Trees die within 3-5 years. Symptoms may appear within 6 months in graft-inoculated plants and warm conditions favor symptom expression [1,2].The disease need to be controlled to maintain the quality and abundance of Mexican lime. Different approaches may be used to prevent, mitigate or control the plant diseases. Application of certain compounds with potential of systemic acquired resistance (SAR) is a control process against pathogen. SAR is a phenomenon whereby a plant activates its own defense against pathogens, wound, etc by prior treatment with plant defense activators such as flavonoids [2,5]. The action mode of plant defense activators against plant pathogens might occur by increasing the peroxidase activity, by the phenolic compounds accumulation, or through inhibition of some antioxidant enzymes and catalases, thereby leading to the production of elevated amounts of H_2O_2 [6,8].

Flavonoids belong to a class of low-molecular-weight phenolic compounds that are widely distributed in the plant kingdom. From the available literature [9,11], it seems that exogenous application of these compounds could enhance the plant resistance against a couple of different biotic and abiotic stresses. Yang *et al*. [9] showed that rutin could enhance resistance to *Pseudomonas*

[*]Corresponding author: Phytochemical Laboratory, Department of Chemistry, Faculty of Sciences, Azarbaijan Shahid Madani University, Tabriz 53714-161, Iran
Email Address: s.mollaei@azaruniv.ac.ir

syringae, *Ralstonia solanacearum*, and *Xanthomonas oryzae* in *Arabidopsis thaliana*, tobacco, and rice, respectively. This enhanced resistance was associated with the expression of genes which involved into SA, reactive oxygen species and nitric oxide signal pathway. Exogenous application of quercetin was studied against *Pseudomonas syringae* in *Arabidopsis thaliana*. The results confirmed quercetin protects plant against bacterial infection through both quercetin-mediated H_2O_2 generation and the involvement of SA and NPR1 [10]. Yiu *et al*. [11] reported the protective effects of exogenously applied catechin on growth, accumulation of proline, lipid peroxidation and activity of antioxidant enzymes in sweet pepper seedlings (*Capsicum annuum* L. cv. Trim Star) exposed to salt stress.

As the ethyl acetate extract of green tea (*Camellia sinensis*) is a source of flavonoids [12], and exogenous application of the extract may induces systemic acquired resistance, thus, the object of the present study was to evaluate the effect of exogenous application of *Camellia sinensis* extract in induction resistance against disease caused by phytoplasma.

Material and Methods

Chemicals

The standard chemicals and HPLC grade solvents were purchased from Merck (Darmstadt, Germany). MilliQ-water was prepared by a MilliQ-System (Mil-lipore, Saint-Quentin-en-Yvelines, France).

Ethyl Acetate Extract of *Camellia sinensis*

To obtain ethyl acetate extract of *Camellia sinensis*,four gram of the dry powdered plant was extracted 3 times with 40 mL of ethylacetate at room temperature for 20 min using ultrasonic bath. Then, the extract was filtered and evaporated under vacuum at 45 °C. Finally, the extracts were hold at 4 °C before the next analyses.

Plant Material and Treatment with Green Tea Extract

Citrus aurantifolia trees (biennial) were obtained from Iranian Research Institute of Plant Protection (IRIPP). The trees were transported intoa 200 m^2greenhouse place. Then, they were treated with the extract of *Camellia sinensis* at the beginning of flowering. The extract was dissolved inabsolute ethanol, then, concentrations of 10, 20, 30, and 40µg/mL were made up with distilled water. The obtained solutions were sprayed on the leaves of the trees. The spray volume was 20 mL per pot. Control groups were sprayed with ethanol/water.After 10 days, 5trees were randomly collected from each treatment for future analysis.

Defense-related Enzyme Assays and Biochemical Substance

The leaves were homogenized in ice-cold K-borate buffer (0.1 M, pH 8.8) containing 2 mM 2-mercaptoethanol and centrifuged at16,000 × g, 10 min, 2 °C and then the supernatants were used to analyze the enzyme activities of PAL, CHS, STS,POD, CAT, and the content of malondialdehyde (MDA) and H_2O_2.

PAL activity was determined by the cinnamic acid and colorimetric method. briefly, 0.5 ml of crude enzyme,1 mL of extraction buffer (without 2-mercaptoethanol) and 0.5 ml of phenylalanine solution were mixed and incubated at 37 °C for 1 hour. The reaction wasstopped with ice bath, and the optical density was measured at 290 nm and was expressed as units of PALper 1hour, per gram cells. Samples withbuffer instead of crude enzyme were used as blank control [13].

A similar procedure was followed for the assessment of chalconesynthase (CHS) and stilbene synthase (STS) activities, using malonyl-CoA and *p*-coumaryl-CoA as precursors which are converted to naringenin and resveratrol, respectively [14]. Products of CHS and STS were measured at 300 nm and were respectively expressed as units of CHS and STSper 1h, per grcells.

The peroxidase (POD) activity was measured using the ultraviolet spectrophotometry methodthrough the reaction whichinitiated by adding enzyme extract to the of mixture (3 mL) containing 50 mM sodium acetate buffer (pH 5.0), 40 mM H_2O_2, 20 mM guaiacol, and appropriate amounts of enzyme extract and was expressed as changes in the absorbance at 470 nm (Cintra 6, GBC, Australia) per mg of protein [15].

For the measured of CAT activity, briefly, 0.1 ml of crude enzyme was added to the mixture of 0.6 ml of 30% hydrogen peroxide and 0.1 mol/L of sodium phosphate buffer (pH 7.0) to start the reaction. The decrease in absorbance was recorded followed by the decomposition of H_2O_2 at 240 nm per min per mg protein [15].

MDA level was assayed by thiobarbituric acid (TBA) method. Briefly, samples were homogenized in trichloroacetic acid solution (10% w/v) and after filtration; the supernatant was added to 0.5% TBA and heated in water bath for 30 min. The amount of MDA was determined from the absorbance at 532 nm followed by correction for the non-specific absorbance at 600 nm and calculated from the extinction coefficient155 mM^{-1} cm^{-1} [15].

The H_2O_2 content was assayed according to the method described by Velikova *et al*. [16]. Briefly, the cells (0.2 g) were homogenized with 5mL of 0.1% (w/v) TCA in an ice bath. The homogenate was centrifuged at 12,000 ×g for 10min, and 0.5 mL of the supernatant was mixed with 0.5 mL of 10 mM potassium phosphate buffer (pH 7) and 1 mL of 1 M KI. The absorbance of the solution was read at 390 nm using spectrophotometer and H_2O_2 content was calculated using a standard curve with concentrations ranging from 0.1 to 1 mM.

Measurement of phenolic contents

Phenolic compounds were quantified using Folin-Ciocalteu method [17]. Briefly, mixture of 0.5 mL methanol extract with 0.5 mL 0.1 N Folin-Ciocalteu reagents (Sigma, USA) was kept 2-5 min, followed by the addition of 1.0 mL of 20% Na_2CO_3. After 10 min of incubation at ambient temperature, the mixture was centrifuged at 12,000 × g for 8 min. The absorbance of the supernatant was measured at 730 nm. The results were expressed as gallic acid equivalents per milliliter extracts.

Total flavonoids were estimated using quercetin as a standard. Briefly, 200 mg fresh cell mass was ground in 3 mL of MeOH: AcOH (99:1) and the homogenate was filtered. One mL of diluted sample was mixed with1 mL of 2% aluminum chloride (methanolic solution). After incubation at room temperature for 15min, absorbance of the reaction mixture was measured at 430 nm [18].

Anthocyanins were extracted overnight from samples with MeOH and 1% HCl at 4 °C and diluted with acidified methanol, and the absorption was measured at 530 nm. Anthocyanin concentration was calculated using an extinction coefficient of 33000 M^{-1} cm^{-1} [15].

Phenolic acids (caffeic acid derivatives) were quantified using Arnow's method [19]. At first, 0.2 ml of methanolic extracts was diluted up to 1 ml volume and added to a mixture containing 1 ml of 0.05N HCl, 1 ml of Arnow's reagent, 10 ml of water and 2 ml of 1N NaOH. Finally, the absorbance was recorded at 515 nmafter 5 min. Evaluation is made with the help of the calibration curve of caffeic acid (k=3.62).

Analysis of phenolic acids using HPLC

The analyses were carried out using an HPLC system (Kenauer, Germany) with K-1001 pump (Knauer, Germany), K-2008 PDA detector (Kenauer, Germany), a manual injection valve (Rheodyne, USA) with a 20 µL loop, and degasser. A gradient elution was performed on a C18 Eurospher column (250 × 4.6 mm, 5 µm). The mobile phase consisted of two different solutions, including solvent A, Water: TFA (100: 0.02 v/v%), solvent B, methanol: TFA (100: 0.02 v/v%). The separation was done by a gradient elution program as follows: The initial mobile phase composition was 75% A for 5 min, followed by a linear gradient to 70% A in 5 min; 10–16 min, from 70 to 55% A; 16–25 min, from 70 to 20% A; 25-30 min was constant. The post-running time was 5 min.

Statistical Analysis

The experiment was carried out using a randomized complete design (RCD) considering five replications for each sample. The data were analyzed by Statistical Analysis System (SAS) software 9.2and all values are shown as the mean ± SD. Statistical analysis was performed using Student´s T-test and the differences between treatments were expressed as significant at level of $p \leq 0.05$.

Results

Effect of Extract on Defense-related Enzyme

To elucidate the mechanism of induced resistance in the extract-pretreated *Camellia sinensis* leaves, the activity of PAL, CHS and STS enzymes, which are known as disease resistance enzymes, was investigated. According to the results (Fig. 1A), The PAL activity of the leaves pretreated with 10, 20, 30, and 40 µg/mL of the extract were 0.38 ± 0.03, 0.36 ± 0.03, 0.04 ± 0.01, and 0.01 ± 0.005Unit/(h × mg protein), respectively. In comparison with the control (0.10 Unit/(h × mg protein), a significant increase in PAL activity was noticed in in the leaves treated with 10 and 20 µg/mLof the extract, while applying exogenous extract up to 20µg/mLreduced significantly (p<0.05)the activity of thisenzyme in theleaves(Fig. 1A).The trend of STS activity was similar with PAL activity, and the statistical analysis (p<

0.05)indicated that the STS activities in different pretreatment were significantly different, and the highest (0.65 ± 0.03mUnit/(h × mg protein)) activity belonged to the leaves pretreated with 20 µg/mL of extract (Fig. 1B). Dissimilar tendencies were observed in the CHS activity and highest activity (0.22 ± 0.03mUnit/ (h × mg protein)) was observed when the leaves were pretreated with 10 µg/mL extract, and then the activity was declined compared to the control (Fig. 1C).

Effect of Extract on Physiological Response

With respect to the results (Fig. 2A), the POD activities were 394 ± 43, 732 ± 53, 986 ± 55, 455 ± 40, and 211 ± 32ΔAbs 470/mg protein, in the control and the leaves pretreated with 10, 20, 30, and 40 µg/mL of the extract, respectively. As shown, in comparison with the control, the POD activity was significantly induced when the leaves were pretreated with 10 µg/mL extract, and then peaked at 20 µg/mL extract (Fig. 2 A).In addition, compared with the control, the POD activity was not significant when the leaves were pretreated with 10 µg/mL extract, while applying exogenous extract up to 30 µg/mL significantly ($p< 0.05$) declined the POD activity from 394 ± 43 to 211 ± 32 (Fig. 2A). Also, the trend of CAT activity was similar with POD activity, and the highest (45 ± 2.56ΔAbs 240/mg protein) and lowest (3 ± 0.56ΔAbs 240/mg protein) activities belonged to the leaves were pretreated with 20 and 40 µg/mL of the extract compared with the control (Fig. 2B).In the leaves that were pretreated with 10 and 20 µg/ml extract, the content of MDA significantly decreased from 71 ± 5 to 41 ± 4 and 28 ± 3 µM MDA/g FW, respectively (Fig. 2C). Exposure to higher concentrations (30 and 40 µg/mL) of extract significantly increased the rate of peroxidation of membrane lipids from 71 ± 5 to 164 ± 10 and 233 ± 11 µM MDA/g FW, respectively (Fig. 2 C). In comparison with the control, the content of H_2O_2 in the leaves significantly ($p< 0.05$)increased from 24 ± 1.4 to 37 ± 2.8, 63 ± 6.1, and 73±6.4 nmole/g FW, when the leaves were pre-treated with 20, 30, and 40 µg/mL of extract supply, respectively(Fig. 2 D). These are consistent with this hypothesis that induction of POD and CAT activities and the decrease of the MDA and H_2O_2 amount are associated with induced resistance in the leaves against phytoplasma.

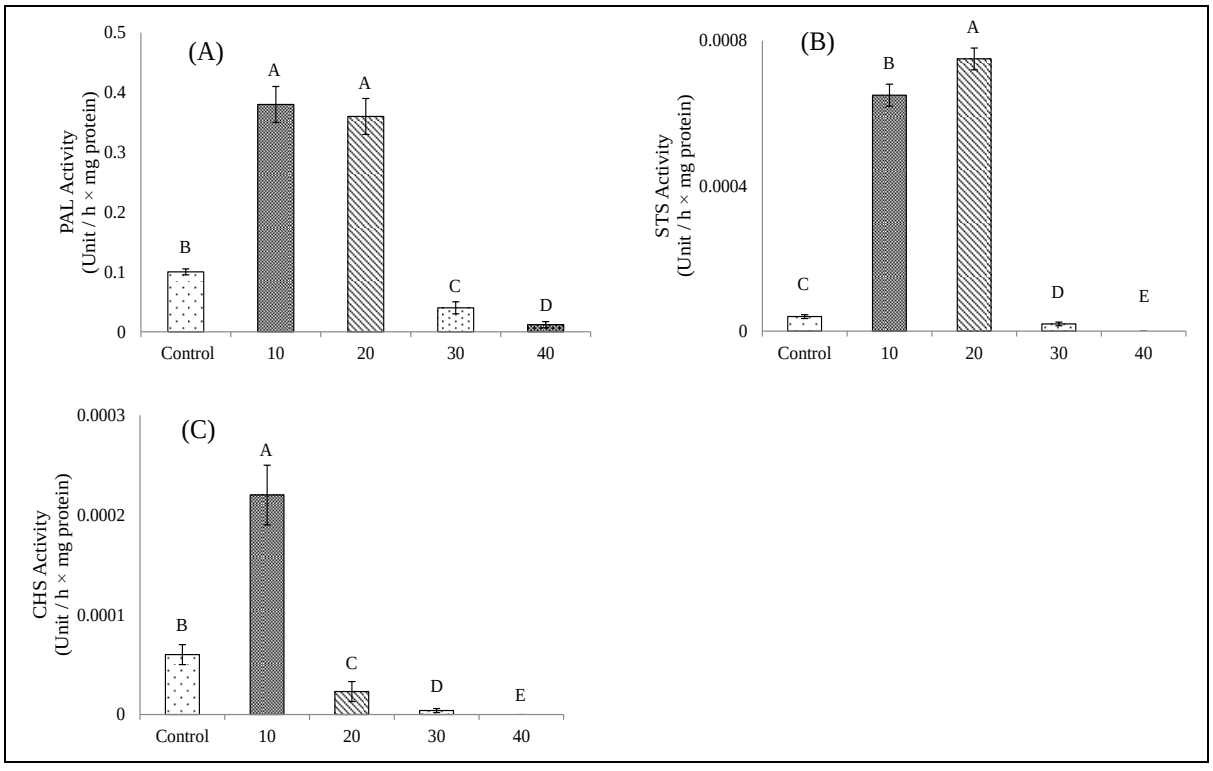

Fig. 1 Effect of different concentrations(µg/mL) of *Camellia sinensis* extract on A) PAL, B) STS and C) CHS activities in Mexican lime cells under WBDL stress.

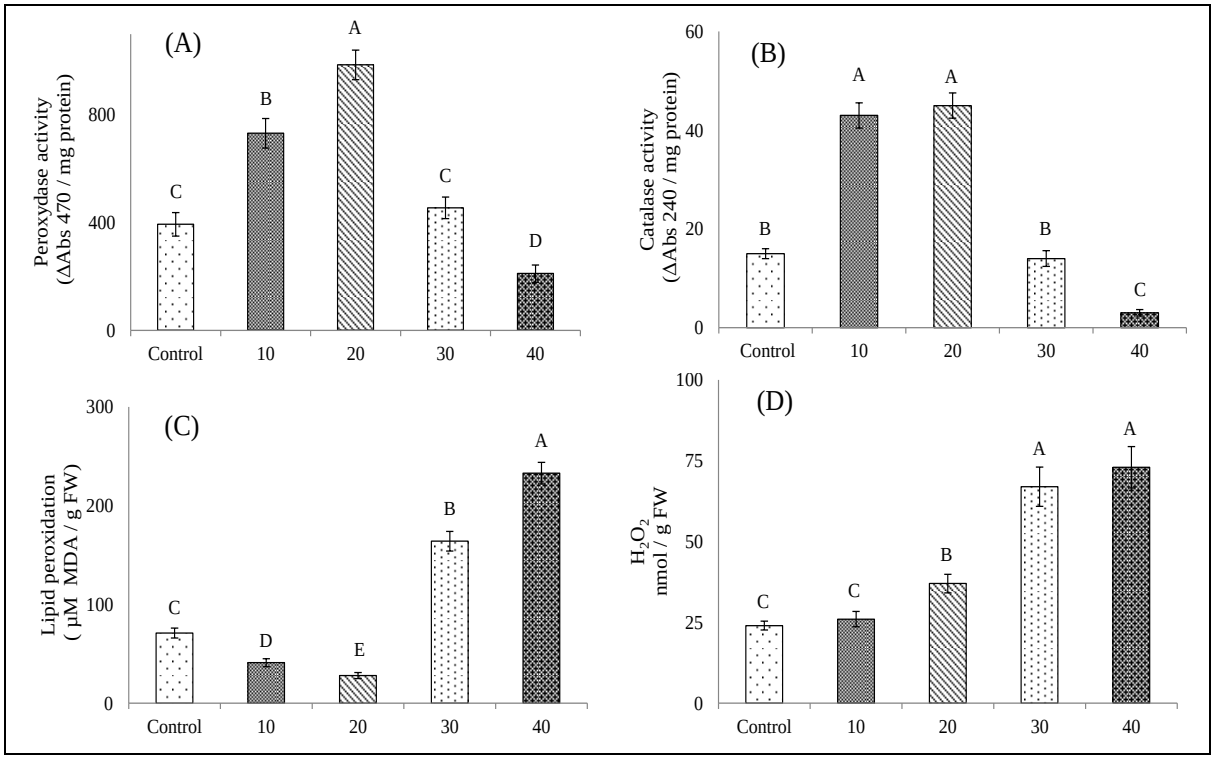

Fig. 2 Effect of different concentrations(µg/mL) of *Camellia sinensis* extract on A) POD, B) CAD activities and the amount of C) MDA and D) H_2O_2 in Mexican lime cells under WBDL stress.

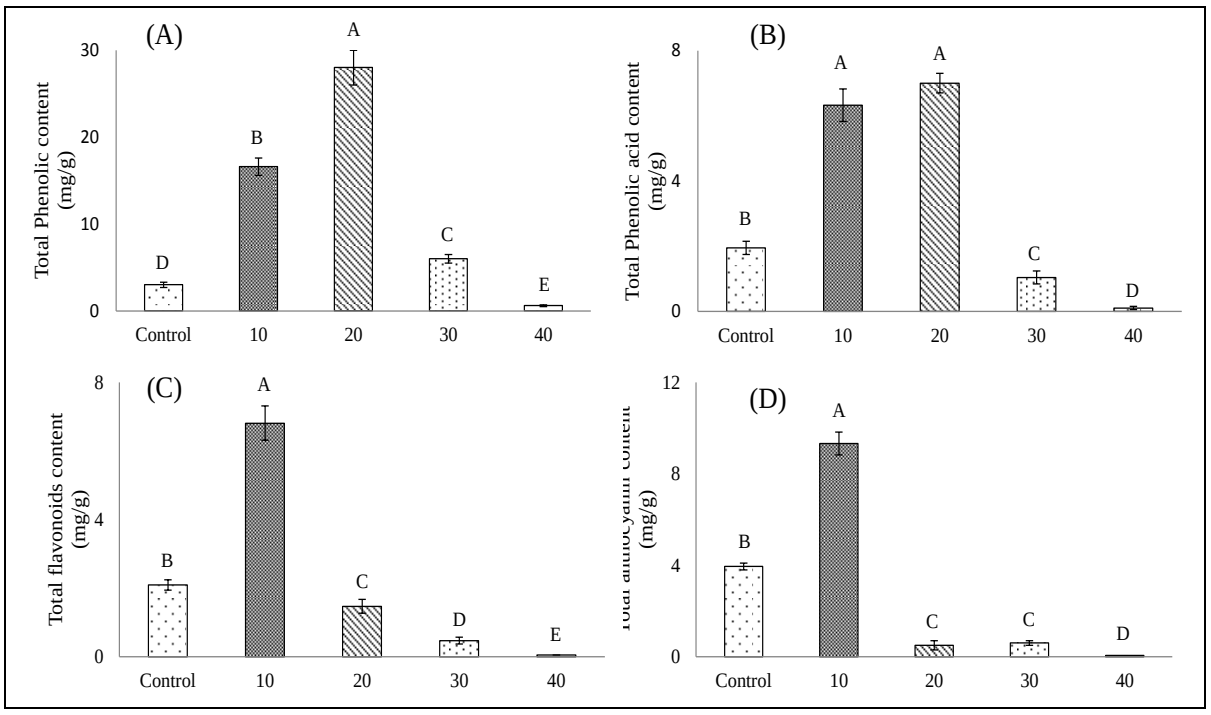

Fig. 3 Effect of different concentrations (µg/mL) of *Camellia sinensis* extract on the amount of A) Total Phenolic (TP), B) Total Flavonoids (TF), C) Total Anthocyanin (TAC) and D) Total Phenolic Acids (TPA) in Mexican lime cells under WBDL stress.

Effect of Extract on Phenolic Compounds

Fig. 3 shows the contents of phenolic compounds of the leaves after exposure to different concentrations of the extract. As shown in Fig. 3A, phenolic compounds were significantly accumulated from 3 ± 0.3 to 16.6 ± 1.0 and 28 ± 2.0 mg/gin the leaves that were pretreated with 10 and 20 µg/mL of the extract, respectively, while, by increasing extract concentration (30 and 40 µg/mL), the amount of phenolic compounds were significantly decreased. The same trend was observed for total phenolic acids (TPA). The amount of phenolic acids increased from 1.94 ± 0.2 to 6.32 ± 0.5 and 7.00 ± 0.3 mg/g when the leaves were pretreated with 10 and 20 µg/mL of the extract, respectively, but the increasing extract concentration (up to 30 µg/mL)decreased significantly the amount of TPA (Fig. 3B). Total Flavonoids (TF) and Total Anthocyanin (TAC) showed approximately different trend compare with TP and TPA. Data presented in Fig. 3C and D show that pretreatment with 10 µg/mL extract have significantly increased amount of TF and TAC compared with the positive control ($p< 0.05$), while pretreatment with 20 µg/mL extract showed toxic effect on the leaves and statically decreased the amount of TF and TAC. The highest amount of TF and TAC (6.81 and 9.32 mg/g) were observed when the leaves were pretreated with 10 µg/mL of the extract.

After separation of some phenolic acids such as galic acid, vanillic acid, syringic acid, salisilic acid and p-coumaric acid using HPLC (Fig. 4), quantitative analysis of them showed that pretreatment of the leaves with 10 and 20 µg/mLof *Camellia sinensis* extract significantly increased the amount of them (Table 1). These data were agreement with total phenolic acids results.

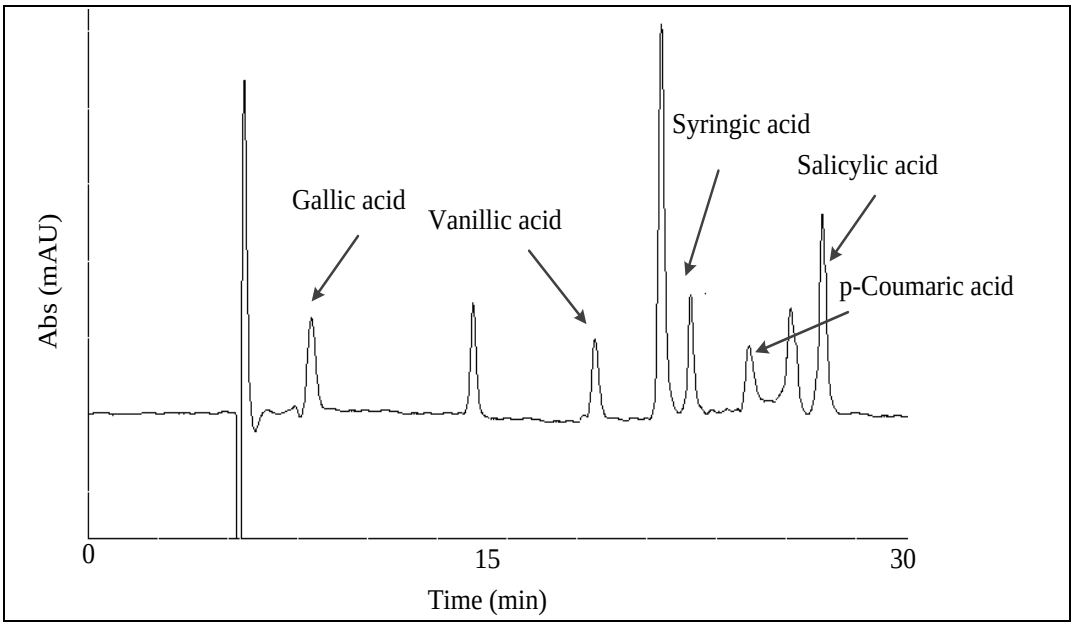

Fig. 4 HPLC chromatogram of phenolic acids standards

Table 1 The amount of phenolic acids (µg/g) in all samples

	Control	*Camellia sinensis* (L.) Kuntze extract (µg/mL)			
		10	20	30	40
Galic acid,	124±8 b	194±6 a	62±2 c	12±1 d	tracee
Vanillic acid,	202±12 b	272±14 a	45±2 c	20±1 d	tracee
Syringic acid,	26±1 b	91±2 a	10±1 c	7±1 d	tracee
Salisilic acid	84±2 b	214±6 a	31±1 c	13±1 d	tracee
p-coumaric acid	101±2 b	154±7 a	15±1 c	9±1 d	1±0e

Control is the leaves pretreated with distilled water. Values are expressed in terms of (µg/g) and given as mean ± SE (n = 3). Means followed by the same letter did not significant differences at $p< 5\%$ (Tukey's test).

Discussion

Plant-derived phenolic compounds such as catechinsare a large group of naturally occurring polyphenols with a wide range of biological activities, including antibacterial, antiviral, insecticidal, and antioxidant activities. Flavonoids are chemically able to prevent oxidation, and their presence/application has been associated with a decrease in oxidative stress markers in living cells. Although formed in normal cell metabolism, higher and uncontrolled production of reactive oxygen species (ROS) leads to extensive oxidation of cell components and irreversible cell/tissue damage underlying the acute pathology of many diseases. Presence of higher amount of flavonoids such as catechin in the leaves of healthy wheat plants has been interpreted as an advantage of the plants against *Puccini triticina* [20]. The study showed that when the lime trees are infected by *Phytoplasma aurantifolia*, the plant defense responses was activated with the further growth and development of the pathogen and therefore the expression level of resistance enzymes such as PAL and CHS increases, followed by biosynthesis of catechin and epicatechin in the leaves [20]. Effects of catechins against gram positive bacteria have been reported and damage of their lipid bilayers have been considered as a mechanism of catechins bactericidal function [21]. Sub-inhibitory concentrations of epigallocatechin gallate and (-)-epicatechin gallate can suppress the expression of bacterial virulence factors and can reverse the resistance of the opportunistic pathogen *Staphylococcus aureus* to β-lactam antibiotics [22].

In the present study, exogenous application of *Camellia sinensis* extract at limited concentrations (less than 20 µg/mL) increased the activity of CAT and POD enzymes in the leaves. Interestingly, it was accompanied by stable production of H_2O_2 and maintenance of their membrane integrity. In higher supply of the extract however, the amount of H_2O_2 and MDA were increased, probably due to reduction in POD and CAT activities. Probably, at the low concentration of the extract, the leaves induced more defense enzymes as a general rule. But at high concentration, there was toxic metabolite production by the extract, and thereby the activity slows down. On the other hand, in the leaves pretreated with the extract, the activity of POD and CAT was higher than control. Bais *et al*. [23] showed the application of minimum inhibitory concentrations of catechin *in vitro* to *A. thaliana* roots triggers a wave of reactive oxygen species initiated at the root meristem, which leads to a Ca^{2+} signaling cascade and triggers genome-wide changes in gene expression, ultimately resulting in root death. Also, Prithiviraj *et al*. [24] suggested that the defense-inducing effect of flavonoids is concentration dependent, as at higher concentrations is phytotoxic. The effects of aqueous garlic bulb extract on the antioxidant defense system of *Solanum melongena* L. confirmed that high spraying caused lipid peroxidation and induced stress in the *Solanum melongena* seedlings [25].

So, the obtained results might be due to the fact that when the leaves challenged with the extract, they ought to secrete more phenol oxidase enzyme for defense.But, as the extract had flavonoid-like compounds such as catechins, the high concentrations of the extract deterred the enzymes activity and caused in a falling off of enzyme activity.

Besides enzymatic antioxidants, flavonoids and other polyphenolic compounds have powerful antioxidant effects or can act as pro-oxidants. One of the main key enzymes involved in the flavonoid biosynthesis is CHS [12] (Fig. 5). CHS catalyzes the condensation of p-coumaroyl-CoA with three C2 units from malonyl-CoA producing a naringenin chalcone [26]. This enzyme plays an important role during the early evolution of land plants by providing protection against various environmental stresses e.g., pathogens attack [27]. Activity of CHS was assay in this condition and the results were coordinated with the amount of Total Flavonoids (TF) and Total Anthocyanin (TAC). Flavonoids and anthocyanin share most of their biosynthesis pathway with stilbenoids starting from *p*-coumaryl CoA and are catalyzed by STS [12] (Fig. 5). Stilbenoids play a role as phytoalexin and also serve as antifungal components [28]. Treatment of the leaves with 10µg/Ml of the extract significantly increased the activities of PAL, CHS, and STS. Subsequently, the contents of all types of phenolic compounds including anthocyanin, flavonoids, phenolic acids, and stilbenes were increased in this treatment.

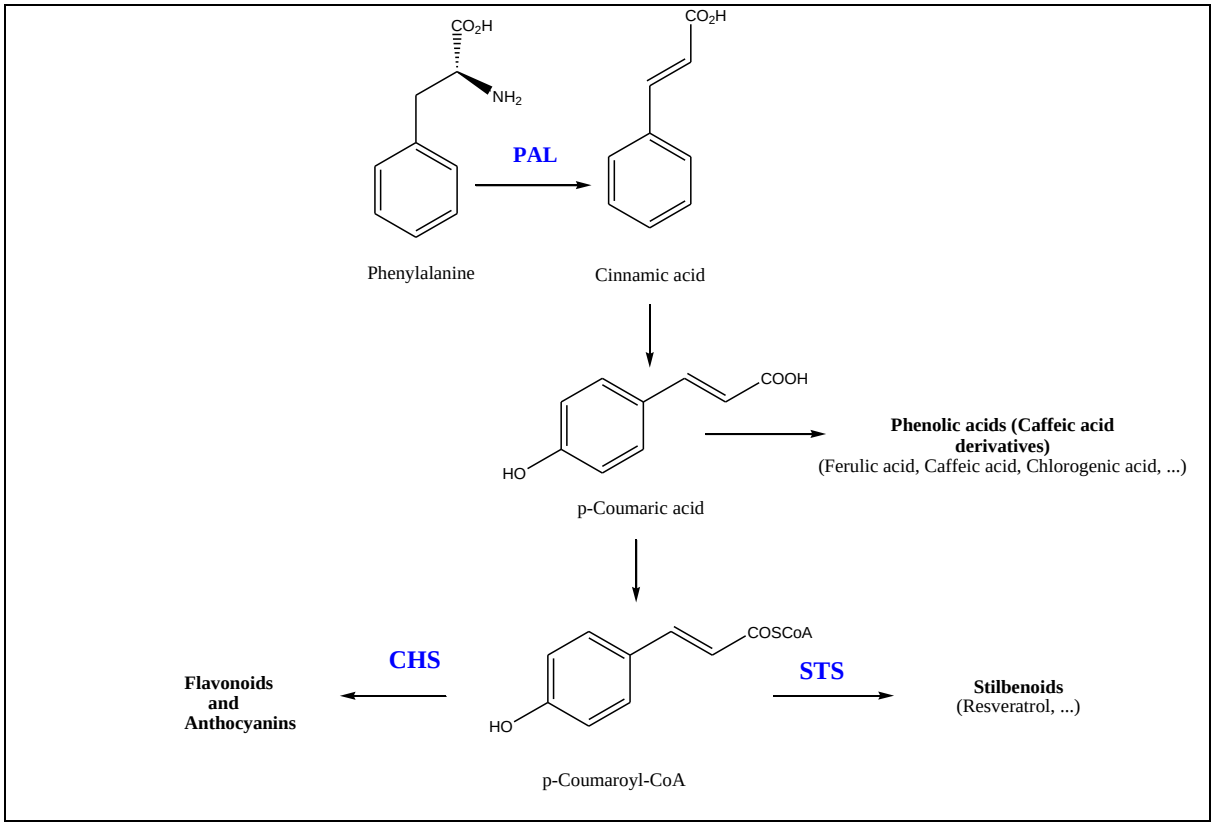

Fig. 5 Biosynthesis pathway of phenolic compounds by showing key enzymes [12].

In concentration of 20 µg/mL of the extract however, the activity of CHS as well as the amounts of anthocyanins, flavonoids decreased, while the activity of STS and corresponding contents of resveratol increased, suggesting a shift in phenylpropanoid biosynthesis from flavonoids to stilbenoids production. Higher concentrations of extract, which containing large amounts of catechins, suppressed the activities of all above mentioned enzymes and their corresponding products.

Five plant extracts were tested as inducers to protect wheat against leaf rust infection caused by *Puccinia triticina*. The tested plant extracts increased chlorophyll, phenol contents, and oxidative enzymes activities of POX and PPO that are responsible for protecting wheat from leaf rust infection [29]. Kumar *et al*. [30] indicated that the spray of potato plants with the extract of *Lantana camara* increased resistance through the accumulation of various phenolic compounds and decreased the disease severity. Hanafy *et al*. [31] showed spraying *Schefflera arboricola* plants with the extract of garlic increased leaf area. The effects of aqueous garlic bulb extract on the growth and antioxidant defense system of *Solanum melongena* L. was investigated [25]. A single spraying modulated the oxidative and antioxidative enzymes in *Solanum melongena* to enhance their growth, whereas spraying three times caused lipid peroxidation and induced stress in the *Solanum melongena* seedlings [25]. Prithiviraj *et al*. [24] showed that flavonoid compounds when administered at lower than MIC concentration could induce growth and a disease protecting phenotype in *A. thaliana* against DC3000. They also suggested that the defense-inducing effects of flavonoids are concentration dependent, as at higher concentrations is phytotoxic [24]. These observations are in agreement with our reports where higher concentrations of tea-derived compounds negatively affected on the antioxidative enzymes as well as phenolic compounds. It seems to be quite natural that all plants are enriched with defense enzymes and they are quiescent in nature and require the appropriate stimulation signals to activate them. It has been reported that biocontrol agents activate latent plant defense mechanisms in response to pathogen infection [8]. These actions might occur by increasing the peroxidase activity, by the phenolic compounds accumulation, or through inhibition of some antioxidant enzymes and catalases, thereby leading to the production of

elevated amounts of H_2O_2 [6, 7]. In conclusion, the current study indicates that inducing the plant's own defense mechanism by applying low concentration of *Camellia sinensis* extract (less than 20 µg/mL), which contains flavonoid-like compounds such as catechin, epicatechin, and epicatechin gallate, can be a novel strategy in plant disease management.

Acknowledgements

The authors gratefully acknowledge the Azarbaijan Shahid Madani University, Tabriz 53714-161, Iranfor financial support of this study**.**

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