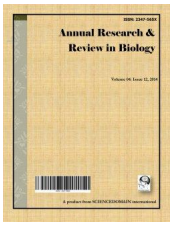

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Salicylic Acid Enhanced Photosynthesis, Secondary Metabolites, Antioxidant and Lipoxygenase Inhibitory Activity (LOX) in *Centella asiatica*

Mohd Hafiz Ibrahim[1*], Hishamuddin Omar[1] and Nurul Amalina Mohd Zain[2]

[1]Department of Biology, Faculty of Science, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Darul Ehsan, Malaysia.
[2]Institute of Biological Science, Faculty of Science, University of Malaya, 50603, Kuala Lumpur, Malaysia.

Authors' contributions

This work was carried out in collaboration between all authors. Author MHI designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors HO and NAMZ managed the analyses of the study. Author NAMZ managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The primary aim of this work was to investigate the impact of foliar application of SA on the leaf gas exchange, the accumulation of secondary metabolites and Lipoxygenase Inhibitory activity (LOX) in medicinal plant *C. asiatica*.
Study Design: The study used randomized complete block design (RCBD) replicated three rwith three replications
Place and Duration of Study: Department of Biology, Universiti Putra Malaysia between January 2014 and March 2014.
Methodology: *Cantella asiatica* was propagated in individual poly bags filled with sand, coco dust and compost in the ratio of 1:1:1 and chicken dung (50 g) per poly bag. The plant was left for a month in a nursery to be acclimatized until they were ready for SA treatment. After a month, the

**Corresponding author: E-mail: mhafiz_ibrahim@upm.edu.my;*

plants were sprayed with two concentrations (1 µM and 10 µM) of salicylic acid solution (SA). Control plants (0 µM) were sprayed with same solution but without SA. The leaf gas exchange, total flavonoid, saponin, Glutathione (GSH), Oxidized Glutathione (GSSG), Phenyll alanine lyase (PAL), Chalcone synthase activity (CHS) and Lipoxygenase inhibitory activity (LOX) was measured.

Results: It was found that production of secondary metabolites (total flavonoids and saponin), GSH, GSSG, PAL, CHS and LOX were solely contributed by interaction of SA levels and plant parts in the descending manner came in leaf treated with 10 µM SA> leaf-1 µM SA>leaf 0 µM SA> stem 10 µM SA> stem 1 µM SA> stem -0 µM SA> root 10 µM SA> root-1 µM SA > root 0 µM SA. High concentration of salicylic acid application (10 µM) was found to be more effective in enhancing the leaf gas exchange traits compared to low concentration (1 µM). Interestingly, it was found that increase in net photosynthesis have a high significant correlation with total flavonoid, total saponin, GSH, GSSG and LOX, indicating that up-regulation of photosynthetic capacity of *C. asiatica* treated with SA, enhanced the production of secondary metabolites, antioxidant and Lipoxygenase inhibitory activity (LOX). This study also showed that there was involvement of CHS and PAL activity in an increase in the production of secondary metabolites and antioxidant activities under SA application.

Conclusion: It was found that SA enhances photosynthetic capacity of *C. asiatica*. This might be due to increase in PAL and CHS activity that was observed during the study. Furthermore, the production of secondary metabolites, antioxidant enzyme and Lipoxygenase inhibitory activity was elevated during the foliar SA application.

Keywords: Salicylic acid; medicinal plants; secondary metabolites; glutathione; chalcone synthase (CHS); phenyl alanine lyase (PAL); lipoxygenase inhibitory activity (LOX).

1. INTRODUCTION

Centella asiatica, commonly known as "Gotu kola, Asiatic pennywort, Indian pennywort, Indian water navelwort, wild violet, and tiger herb" in English, is a tropical plant, which has been also cultivated successfully due to its medical importance, and it has a long history of utilization in ayurvedic and Chinese traditional medicines since centuries. *Centella asiatica* is a stoloniferous medicinal herb which grows in tropical and subtropical regions. It has served in traditional medicine in the therapy of various physical and mental ailments presumably since prehistoric times. Several studies are reported on the antioxidant [1,2], anti-inflammatory [3], neuroprotective [4-6] and cardioprotective [7,8] activities of this plant. The properties are caused by secondary metabolites compounds in the plants. The major chemical class found in this plant is triterpene sapono-sides, asiatic acid, madecassic acid (6-hydroxy-asiatic acid), asiaticoside, madecassoside, and madasiatic acid.

Flavonoids and other phenolic acids are considered to be responsible for the wide spectrum of pharmacological activities attributed to the herb [9]. Flavonoids are polyphenolic compounds that contain a C-15 flavone skeleton (diphenylpropane). They consist of flavones, flavonols, flavanols, flavanone and flavanonols, and constitute the majority of plant secondary metabolites [10]. The concentration of total flavonoids and phenolics metabolites was found to be affected by environmental conditions such as light intensity, carbon dioxide levels, temperature, fertilization, and biotic and abiotic factors, which can change the concentration of these active constituents [11].

The use of plant elicitor can usually enhance the production of plant secondary metabolites. Plant elicitors refer to chemicals that can trigger physiological and morphological responses and secondary metabolism in plants [12]. The uses of the plant elicitor causes a variety of defensive reactions and the accumulations of secondary metabolites in plants [13,14]. Generally, plant secondary metabolites consist of flavonoids and phenolics compounds are unique sources for pharmaceuticals, food additives, flavors, and other industrial materials either as part of a final product or as a raw material [15]. To enhance the production of these secondary metabolites, strategies such as treating with elicitors and invoking biotic and abiotic stresses have been used in plants [16].

One of the elicitors known is salicylic acid (SA). This plant elicitor plays a key role in a plant's growth, development, and defense responses, and it is involved in some signal transduction systems to induce particular enzymes [17]. As an

elicitor, SA regulates the PAL enzyme activity, which as a biosynthetic enzyme catalyzes biosynthetic reactions for forming defensive compounds [18,19] and SA regulates the antioxidant scavenger enzymes such gluthatione (GSH), which together increase a plant's tolerance to oxidative stresses [20-22]. Salicylic acid has been identified as one of the important phenolic compound in plants and also reported as allelopathic chemical [23,24]. The results of previous studies showed that production of soluble carbohydrates, sugars and secondary metabolites enhanced in plants exposed to SA [25]. Some researcher [26] reported positive effects of SA application were correlated a with significant increase in total soluble proteins, flavonoids, antioxidant, total soluble carbohydrates and sugars in pea (*Pisum Sativum* L.) seeds.

2. MATERIALS AND METHODS

2.1 Experimental Location, Plant Materials and Treatments

The experiment was carried out under a growth house complex at Ladang 2, Faculty of Agriculture Glasshouse Complex, Universiti Putra Malaysia (longitude 101° 44' N and latitude 2° 58'S, 68 m above sea level) with a mean atmospheric pressure of 1.013 kPa. The growth houses receiving 12-h photoperiod and average photosynthetic photon flux density of 300 µmol/m^2/ s. Day and night temperatures were recorded at 30 ± 1.0 °C and 20 ± 1.5 °C, respectively, and relative humidity at about 70% to 80%. Vapor pressure deficit ranged from 1.01 to 2.52 kPa. *Cantella asiatica* were propagated in individual polybags filled with sand, coco dust and compost in the ratio of 1:1:1 and chicken dung (50 g) per poly bag. The ingredients of the organic fertilizer include N (1.5%), P (1.0%), K (0.9%), Mg (0.1%), Zn (0.1%) and Bo (0.5%). The plant was left for a month in a nursery to acclimatize until they were ready for the treatments. After a month, they were sprayed with two concentrations (1 µM and 10 µM) of salicylic acid solution (SA; 2-hydroxybenzoic acid + 100 µl dimethyl sulfoxide + 0.02% Polyoxyethylenesorbitan monolaurate, Tween 20, Sigma Chemicals; pH 6.5; 5 ml/plant). Control plants were sprayed with same solution but without SA [27,28]. Before the experiment initiated, preliminary experiment was conducted to determine the optimum concentration of Salicylic Acid to be applied to *C. asiatica*. The concentration of SA from 0– 30 µM was used. Among the treatments, the application of ABA below 11 µM has shown to significantly increased the plant dry weight and leaf number, however concentration more than 11 µM have shown to reduce the plant dry weight and leaf number. Hence the concentration < 11 µM SA was used in this study. This experiment was arranged in randomized complete block design with SA application (0 µM, 1 µM and 10 µM) and plant parts (leaves, stems, roots) are the factors and replicated three times. Each of the experimental unit contained 5 plants, totaling 135 plants used in the study.

2.2 Leaf Gas Exchange Measurement

The measurements were obtained using a closed infra-red gas analyzer LICOR 6400 Portable Photosynthesis System (IRGA, Licor. Inc. Nebraska, NE, USA). Prior to use, the instrument was warmed for 30 min and calibrated with the ZERO IRGA mode. Two steps are required in the calibration process: first, the initial zeroing process for the built-in flow meter; and second, zeroing process for the infra-red gas analyzer. The measurements used optimal conditions set at 400 µmol mol−1 CO2, 30°C cuvette temperature, 60% relative humidity with air flow rate set at 500 cm3 min−1, and modified cuvette condition of 800 µmol m^{-2} s^{-1} photosynthetically photon flux density (PPFD). Measurements of gas exchange were carried out between 09:00 to 11:00 a.m. using fully expanded young leaves numbered three and four from the plant apex to record net photosynthesis rate (A). The operation was automatic and the data were stored in the LI-6400 console and analyzed by "Photosyn Assistant" software (Version 3, Lincoln Inc., Columbus, OH, USA). Several precautions were taken to avoid errors during measurements [29]. About 40 plants were used as replicates in each SA treatments.

2.3 Total Flavonoids Quantification

The method of extraction and quantification of total flavonoids contents followed after Ibrahim et al. [30]. For total flavonoids determination, a sample (1 mL) was mixed with $NaNO_3$ (0.3 mL) in a test tube covered with aluminium foil, and left for 5 minutes. Then 10% $AlCl_3$ (0.3 mL) was added followed by addition of 1 M NaOH (2 mL). Later, the absorbance was measured at 510 nm using a spectrophotometer with rutin as a standard (results expressed as mg/g rutin dry sample).

2.4 Total Saponin Content

Total saponin content was determined according to Makkar et al. [31] based on the vanillin-sulfuric acid colorimetric reaction. The results were expressed as mg diosgenin equivalent per gram dry matter of the plant material.

2.5 Phenylalanine-ammonia-lyase (PAL)

Phenylalanine-ammonia-lyase (PAL) activity was measured using the method described by Martinez and Lafuente [32]. The enzyme activity was determined by measuring spectrophotometrically the production of trans-cinnamic acid from L-phenylalanine. Enzyme extract (10 µL) was incubated at 40°C with 12.1 mM L-phenylalanine (90 µL, Sigma) that were prepared in 50 mM Tris-HCl, (pH 8.5). After 15 minutes of reaction, trans-cinnamic acid yield was estimated by measuring increase in the absorbance at 290 nm. Standard curve was prepared by using a transcinnamic acid standard (Sigma) and the PAL activity was expressed as nM trans-cinnamic acid µg/protein/ hour.

2.6 Chalcone Synthase (CHS) Assay

CHS activity was assayed spectrophotometrically as described in [33]. Enzymes were extracted at 4°C by homogenizing the frozen harvested cells (0.4 g) in 1 mL of 0.1 M borate buffer (pH 8.8) containing 1 mM 2-mercaptoethanol with a homogenizer (Polytron). The homogenates were treated with 0.1 g of Dowex l × 4 for 10 min and the cell debris and resin were removed by centrifugation at 15,000 rpm for 10 min. A 0.2 g sample of Dowex l × 4 resin was added to the supernatant and treated for another 20 min. The resin was then removed by centrifugation at 15,000 rpm for 15 min. The resultant supernatant was used in the CHS assay. The CHS assay was performed with 100 µL of enzyme extract mixed with 1.89 mL of 50 mM Tris-HCl buffer, pH 7.6, containing 10 mM KCN. The enzyme reaction was allowed to proceed for 1 min at 30°C after adding 10 mg chalcone to 10 µL ethylene glycol monomethylether. The activity was determined by measuring the absorbance at 370 nm.

2.7 Measurement of Glutathione (GSH) and Oxidized Glutathione (GSSG)

GSH and GSSG were assayed using the method described by [34]. Total glutathione were determined by reacting plant extracts (0.5 mL) with 50 mM KH2PO4/2.5 mM EDTA. buffer (pH 7.5), 0.6 mM DTNB [5,5-dithiobis-2-nitrobenzoic acid] in 100 mM Tris-HCl, pH 8.0, 1 unit of glutathione reductase (GR, from spinach, EC 1.6.4.2) and 0.5 mM NADPH. GSH was quantified from the reaction mixture by mixing plant extract (0.5 mL) with 60 mM KH2PO4/2.5 mM EDTA buffer (pH 7.5), 0.6 mM DTNB [5,5-dithiobis-2-nitrobenzoic acid] in 200 mM Tris-HCl, pH 8.0. The mixture was incubated at 30°C for 15 min, and the reaction was followed as the rate of change in absorbance at 412 nm using light spectrophotometer (UV-3101P, Labomed Inc, USA) GSSG was determined after removal of GSH from the plant extract.

2.8 LOX Inhibitory Assay

Lipoxygenase (EC 1.13.11.12 type 1-B) (LOX) was assayed according to the method reported by Wu [35]. A mixture of a solution of sodium borate buffer (1 mL, 0.1 M, pH 8.8) and soybean LOX (10 µL, final conc. 8,000 U/mL) was incubated with plant species extract sample (10 µL) in a 1 mL cuvette at room temperature for 5 min. The reaction was initiated by the addition of linoleic acid substrate (10 µL, 10 mmol). The absorbance of the resulting mixture was measured at 234 nm over time at a rate of one measurement/min (3 readings). Inhibition of LOX was assessed using the following equation:

> % Inhibition = 100 × (absorbance of the control −absorbance of the sample)/absorbance of the control)

The effective concentration (µg/mL) at which LOX activity is inhibited by 50% (IC50) was represented in a graph. Nordihydroguaiaretic acid (NDGA) was used as a positive standard.

2.9 Statistical Analysis

Data were analyzed using analysis of variance by SAS version 17. Mean separation test between treatments was performed using Duncan multiple range test and standard error of differences between means was calculated with the assumption that data were normally distributed and equally replicated [36,37].

3. RESULTS AND DISCUSSION

3.1 Leaf Gas Exchange Properties

The leaf gas exchange parameters (net photosynthesis, stomatal conductance, light compensation point, respiration rate and

intercellular CO_2) were influenced by salicylic acid application ($p \leq 0.01$) on *C. asiatica*. Generally net photosynthesis was highest under high application of SA (10 µM) that recorded 8.14 µmol/m^2/s followed by 1 µM (7.11 µmol/m^2/s) and lowest at control that just recorded 3.22 µmol/m^2/s. The same trend was followed in stomatal conductance where at 10 µM SA the stomatal conductance was 35% and 53% higher compared to 1 µM SA and control respectively. It was also found that the light compensation point for 10 µM SA applications was lowest (17.21 µmol/m^2/s) compared to 1 µM SA (20.14 µmol/m^2/s) and 0 µM SA (32.14 µmol/m^2/s). The increase in net photosynthesis of *C. asiatica* under SA application might be due to reduction in

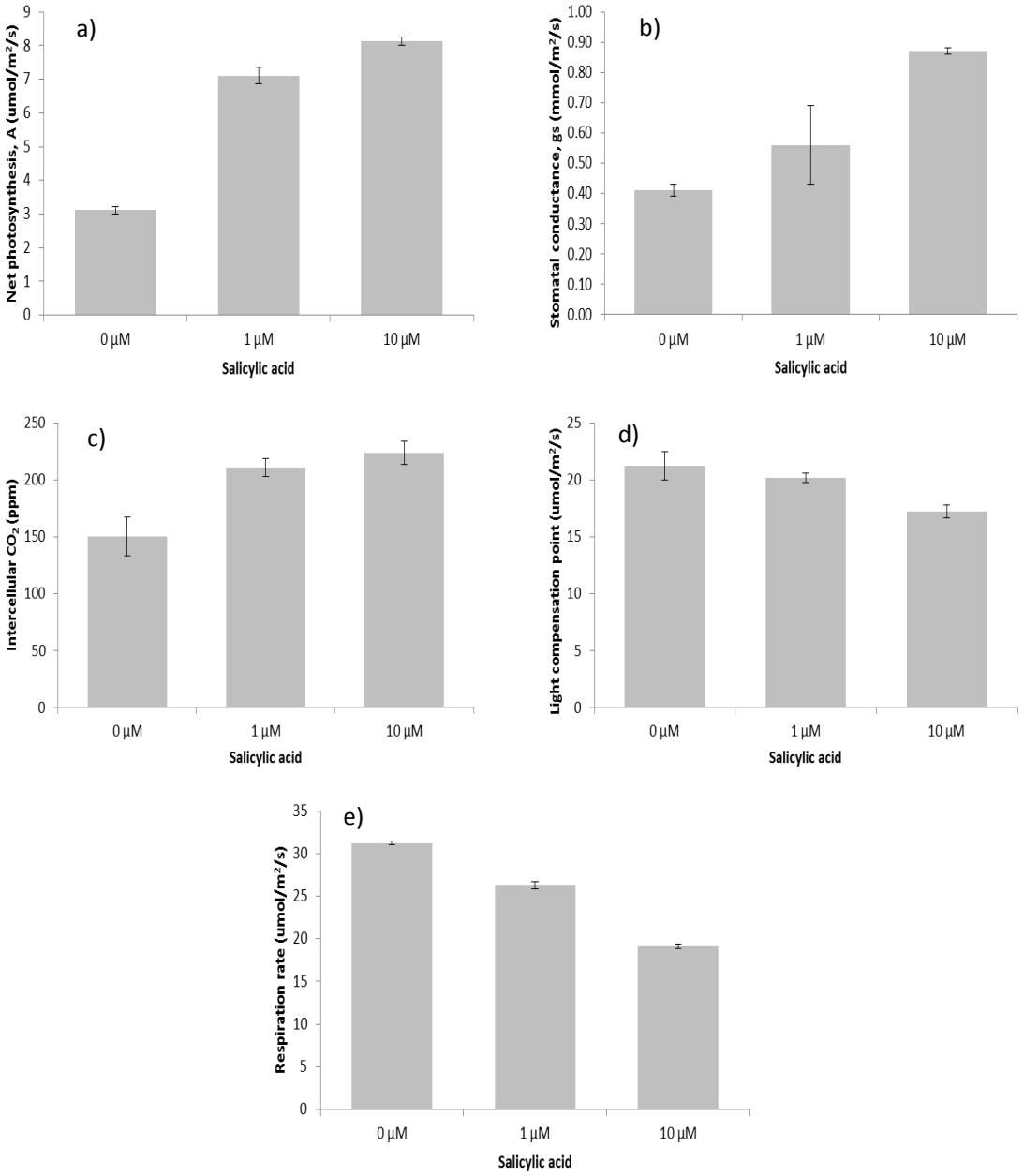

Fig. 1. Impact of foliar salicylic acid on net photosynthesis (a), stomatal conductance (b), intercellular CO_2 (c), light compensation point (d) and respiration rate of *C. asiatica*

N = 15. Bars represent standard error of differences between means (SEM)

respiration rate [38]. It was observed that respiration rate under 10 µM SA was 36% and 63% low compared to 1 µM and 0 µM respectively. This indicate that application of SA can enhanced photosynthetic rate by reducing the respiration rate in C. asiatica. Significant positive correlationship of net photosynthesis was establish between intercellular CO2 (r2 = 0.912; p≤0.05) and stomatal conductance (r2 = 0.876; p≤0.05) implies that increased in net photosynthesis of C. asiatica was due to decreased in stomatal resistance that simultaneously enhanced the uptake of CO_2 in leaf mesophyll (C_i) [39,40]. The result was in agreement with the findings of Ghasemzadeh and Jaafar [41] and Shi et al. [42] where they found that application of SA has increased the net photosynthesis and intercellular CO_2 in ginger and cucumber respectively. The present finding suggest that increased photosynthetic capacity of *C. asiatica* with SA application might be due to increase in stomatal conductance that increase the CO_2 uptake that increased the C_i in leaves, high uptake of CO_2 would reduce the respiration rate that was observed in high application of SA (10 µM).

3.2 Total Flavonoids and Saponin Profiling

Accumulation of total flavonoids and saponin in *C. asiatica* was influenced by the interaction effect between salicylic acid (SA) concentration and plant parts (p≤0.01; Table 1). Generally, total flavonoids was observed to be higher in the leaf at 10 µM SA (1.47 mg rutin/g dry weight) followed by leaf-1 µM SA (1.31 mg rutin/g dry weight), leaf-0 µM SA (0.89 mg rutin/g dry weight), stem-10 µM SA (0.88 mg rutin/g dry weight), stem-1 µM SA (0.76 mg rutin/g dry weight), stem-0 µM SA (0.64 mg rutin/g dry weight), root-10 µM SA (0.51 mg rutin/g dry weight), root-1 µM SA (0.42 mg rutin/g dry weight) and root-0 µM SA (0.31 mg rutin/g dry weight). Total saponin content followed the same trend with total flavonoids where the highest total saponin was observed in leaf at 10 µM SA that registered 11.62 mg diosgenin/g dry weight and the lowest was in the root at 0 µM SA that contained only 4.12 mg diosgenin/g dry weight. The data indicate application of SA at high concentration can enhance the production of secondary metabolites in *C. asiatica*. Data from correlation Table 2 showed that total flavonoids and saponin have a significant positive correlation with phenyalanine ammonia lyase (PAL) activity (Total flavonoids; r^2 = 0.911; p≤0.05; Total saponin; r^2 = 0.934; p≤0.05), that indicates activity of PAL was increased under application of salicylic acid that simultaneously enhanced the production of secondary metabolites in *C. asiatica* (total flavonoid and saponin) [43,44]. The present result showed, that foliar application of SA can up-regulate the production of secondary metabolites. The high flavonoids and saponin content in the plant has been shown to have anticancer properties and also have an application to use as antibiotics, anti-diarrhea, anti-ulcer and anti-inflammatory agents, as well as in the treatment of diseases such as hypertension, vascular fragility, allergies and hyperchlolestrolemia [45,46].

Table 1. Total flavonoids and saponin contents in different parts of *C. asiatica* under different salicylic acid concentration

Salicylic acid	Plant parts	Total flavonoids (mg rutin/ dry weight)	Total Saponin (mg diosgenin /g dry weight)
	Leaves	0.89±0.12^{c}	9.47±0.01^{b}
0 µM	Stems	0.64±0.34^{e}	7.34±0.32^{c}
	Roots	0.31±0.02^{h}	4.12±0.04^{e}
	Leaves	1.31±0.23^{b}	10.61±0.21^{a}
1 µM	Stems	0.76±0.14^{d}	7.39±0.43^{c}
	Roots	0.42±0.12^{g}	5.19±0.21^{d}
	Leaves	1.47±0.02^{a}	11.62±0.03^{a}
10 µM	Stems	0.88±0.34^{c}	9.46±0.05^{b}
	Roots	0.51±0.67^{f}	5.22±0.06^{d}

All analyses are mean ± standard error of mean (SEM), N = 15. Means not sharing a common single letter were significantly different at P ≤ 0.05

Table 2. Pearson correlation between parameters measured during the experiment

		1	2	3	4	5	6	7	8	9	10	11	12	13
1	Photo	1.000												
2	Stomatal	0.883*	1.000											
3	Ci	0.765*	0.453	1.000										
4	LCP	0.554	0.345	0.212	1.000									
5	Respiration	-0.342	0.213	0.098	0.231	1.000								
6	Flavonoids	0.921*	0.123	0.123	0.071	0.021	1.000							
7	Saponin	0.911*	0.211	0.121	0.213	0.123	0.912*	1.000						
8	GSH	0.911*	0.213	0.021	0.134	0.008	0.887*	0.771	1.000					
9	GSSG	0.899*	0.090	0.072	0.142	0.211	0.812*	0.611	0.812*	1.000				
10	GSH/GSSG	0.654	0.070	0.213	0.081	0.032	0.563	0.433	0.023	0.344	1.000			
11	CHS	0.865*	0.213	0.213	0.211	0.143	0.871*	0.876*	0.711	0.231	0.211	1.000		
12	PAL	0.890*	0.142	0.143	0.112	0.123	0.913*	0.912*	0.812	0.012	0.121	0.971*	1.000	
13	LOX	0.711*	0.244	0.152	0.032	0.032	0.934*	0.911*	0.711	0.014	0.009	0.912*	0.981**	1.000

** and ** significant at p≤0.05 and p≤0.01. Note, Photo = net photosynthesis; stomatal = stomatal conductance; Ci = intercellular CO2; LCP = light compensation point; GSH= glutathione; GSSG = oxidized gluthitione; CHS=chalcone synthase; PAL = phenyl alanine lyase activity; LOX = Lipoxygenase inhibitory* activity

3.3 Glutathione (GSH), Oxidised Glutathione (GSSG) and Ratio of GSH/GSSG

The GSH, GSGG and GSH/GSSG in *C. asiatica* were influenced by the interaction between SA and plant parts (p≤0.01; Table 3). Generally, the accumulation of GSH and GSGG followed the same trends where, the highest GSH and GSSG was under 10 µM SA followed by 1 µM SA and control. The highest accumulation in plant parts was observed under leaf followed by stems and the least in the roots. For GSH the highest accumulation of GSH (701.22 nmol gluthathione/g dry weight) was found to be in the leaf at 10 µM SA, while the lowest (401.16 nmol glutathione/g dry weight) was observed in the root at 0 µM SA. In GSSG, leaf-10 µM SA and root-0 µM SA recorded 121.12 and 72.16 nmol oxidised glutathione/g dry weight, respectively. The GSH/GSSG ratio was highest under stem + 10 µM SA (6.02) and lowest in leaves under 0 µM SA (5.31). GSH is a tripeptide composed of cysteine, glutamic acid and glycine and is the most abundant non-protein thiol in the cells. Its active group is the thiol (–SH) of cysteine. The majority of GSH are maintained in the reduced state. The GSH plays an important role in the stabilization of many enzymes. In addition, as antioxidant scavenger it serves as a substrate for dehydroascorbate (DHAsA) reductase and is also directly reactive with free radical including the hydroxyl radical to prevent the inactivation of enzymes by oxidation of an essential thiol group [47]. GSSG consists of two GSH molecules joined by their –SH group into a disulfide bridge and was found to be present in low quantities compared to GSH [48]. In the present study, we found that SA application increased GSH, GSSG and GSH/GSSG ratio. The high GSH and GSH/GSSG ratio are necessary for several physiological functions. Those include activation and inactivation of redox dependent enzyme systems and regeneration of cellular antioxidant ascorbic acid under oxidative conditions [49]. Usually, the increase in GSH and the ratio of GSH/GSSG in application of SA in the present study was associated with an increase in antioxidant properties [50]. In the current study, it was shown that GSH and GSSG have a strong positive relationship with total flavonoids and total saponin. The result showed that the increase in antioxidative properties of *C. asiatica* under SA application might be due to increase in production of total flavonoids and saponin that can increase the antioxidant of this plant under application of exogenous elicitors such as salicylic acid [51,52].

3.4 Chalcone Synthase (CHS) Activity

According to the results, CHS activity was influenced by the SA concentration (p ≤0.01; Fig. 2). It was observed that CHS activities were highest in the leaves compared to the other plant parts. From the Fig. 1 there were no significant differences between the application of 10 µM SA and 1 µM SA in the activities of CHS. In the leaves, the CHS ranged from 2.31- 6.67 nkat/mg protein, stems (1.12 – 4.32-nkat/mg) and roots (1.13- 4.34 nkat/mg). The present result indicated that the application of SA can increase the CHS activity. The CHS activity was highest in the leaf followed by the stems and the roots. The CHS activity was found to have significant positive correlationship with total flavonoid (r^2 = 0.911) and total saponin (r^2 = 0.923). This is basically due to the fact that CHS is a precursor secondary metabolites biosynthesis [53]. It is hypothesized that, the increase in production of total flavonoids and saponin in the present work could be attributed to an increase in CHS activity in SA treated plants. Our results are consistent with [54] who reported application of SA induced CHS activity in beans, [55] pointed out that change in CHS activity rather than PAL activity (also involving enzymes for flavonoid synthesis) was correlated with changes in anthocyanin accumulation under various culture conditions. CHS is the first enzyme to switch from phenylpropanoid metabolism to flavonoid metabolism and is believed to be a key enzyme in this system [56]. These findings together with evidence for channeling between SA concentration and CHS activity in the general phenylpropanoid pathway [57], indicate that the organization of these systems are important in the understanding of how plant metabolism is regulated.

3.5 Phenyalanine Ammonia Lyase (PAL) Activity

Fig. 3 showed that, PAL activity of *C. asiatica* under application of different SA concentration. The effects of PAL are contributed by foliar SA applications (p≤0.05). Generally it was found that the PAL activity was the highest in 10 µM SA application and followed by 1 µM SA and lowest at control (0M SA). In the current study, the highest PAL activity was recorded in the root at 10 µM SA applications that registered 47.16 nM

transcinamic/mg/protein/hour and the lowest in the stems at 0 µM SA application that recorded 21.18 nM transcinamic/mg/protein/hour. Correlation Table 2 showed that PAL has a significant positive correlation with total flavonoid (r^2 = 0.901; p≤ 0.05), total saponin (r^2 = 0.934; p≤ 0.05) and CHS activity (r^2 = 0.934; p≤ 0.05). This indicated that, application of SA to *C. asiatica* has increased the PAL activity that simultaneosly enhanced the production plant secondary metabolites and signaling that PAL and CHS are the key enzyme in regulating the SA elicited flavonoid and saponin accumulation [56]. PAL is a branch point enzyme between the primary and secondary metabolites, and is a key enzyme for regulating the influx of phenylalanine to the biosynthesis of phenolics compounds [54]. Previous studies have shown that the PAL activity could be induced by SA elicitation in parsley [58], citrus [59] and grapes [23] which could result in accumulation of plant secondary metabolites [13]. However, yet very little is known or understood between the role of SA plays in regulating PAL activity and flavonoid compounds biosynthesis [60].

3.6 Lipoxygenase (LOX) Inhibitory Activity

LOX catalyzes dioxygenation of polyunsaturated fatty acids to yield cis, trans-conjugated diene hydroperoxides. LOX is involved in provoking several inflammation-related diseases such as arthritis, asthma, cardiovascular, cancer and allergic diseases [62]. For this reason, targeting inhibitors of LOX is a promising therapeutic target for treating a wide spectrum of human diseases. Results for LOX inhibitory activity (IC_{50}) are shown in Fig. 4. *Cantella asiatica* that been applied with 10 µM SA showed a strongest

Table 3. Gluthathione (GSH), Oxidised Gluthatione (GSSG) and GSH/GSSG ratio in different part of *C. asiatica* under different salycylic acid concentrations

Salycylic acid	Plant parts	GSH (nmol/g dry weight)	GSSG (nmol/g dry weight)	GSH/GSSG
0 µM	Leaves	628.11±26.54^{b}	116.11±12.45^{b}	5.31±0.78^{e}
	Stems	569.31±13.24^{d}	97.16±11.34^{e}	5.96±0.43^{b}
	Roots	401.16±15.54^{g}	72.16±17.43^{h}	5.57±0.98^{c}
1 µM	Leaves	698.31±21.34^{a}	117.31±21.34^{b}	5.96±0.63^{a}
	Stems	579.38±18.11^{d}	100.11±18.22^{d}	5.79±0.56^{b}
	Roots	447.21±12.34^{f}	81.42±15.21^{g}	5.51±0.32^{d}
10 µM	Leaves	701.22±14.54^{a}	121.12±13.21^{a}	5.78±0.31^{b}
	Stems	600.11±11.25^{c}	107.21±11.21^{c}	6.02±0.65^{a}
	Roots	501.21±20.32^{e}	89.11±14.32^{f}	5.63±0.42^{c}

All analyses are mean ± standard error of mean (SEM), N = 15. Means not sharing a common single letter were significantly different at P ≤ 0.05

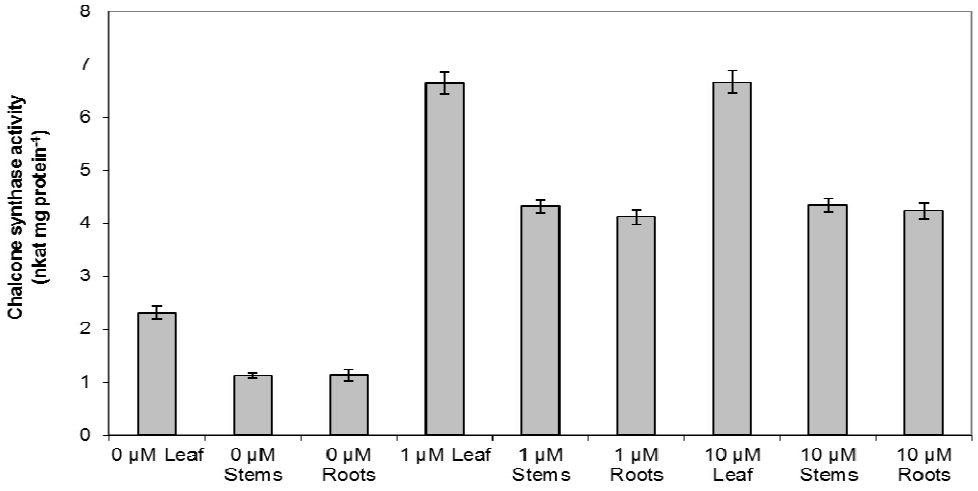

Fig. 2. Impact of foliar salicylic acid on CHS activity of *Centella asiatica*. N = 15. Bars represent standard error of differences between means (SEM)

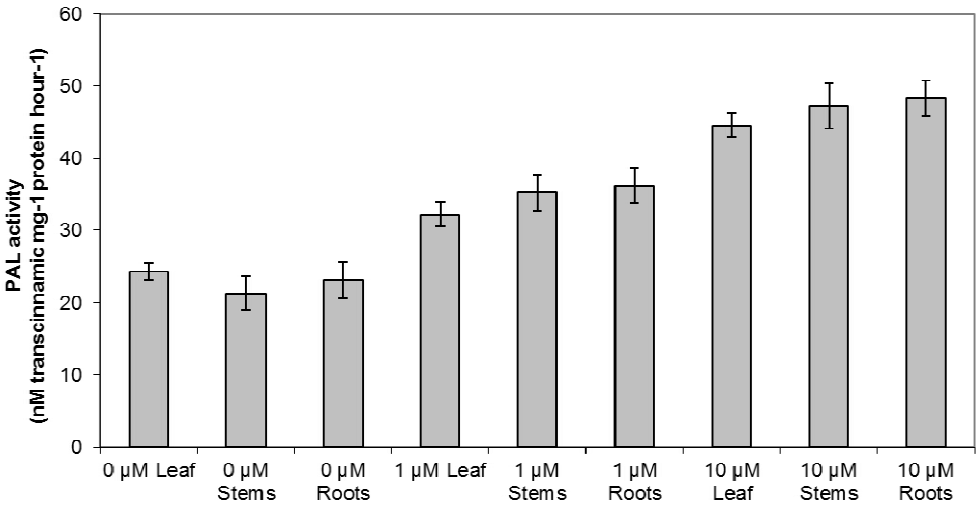

Fig. 3. Impact of salicylic acid on PAL actvity of *Centella asiatica*. N = 15. Bars represent standard error of differences between means (SEM)

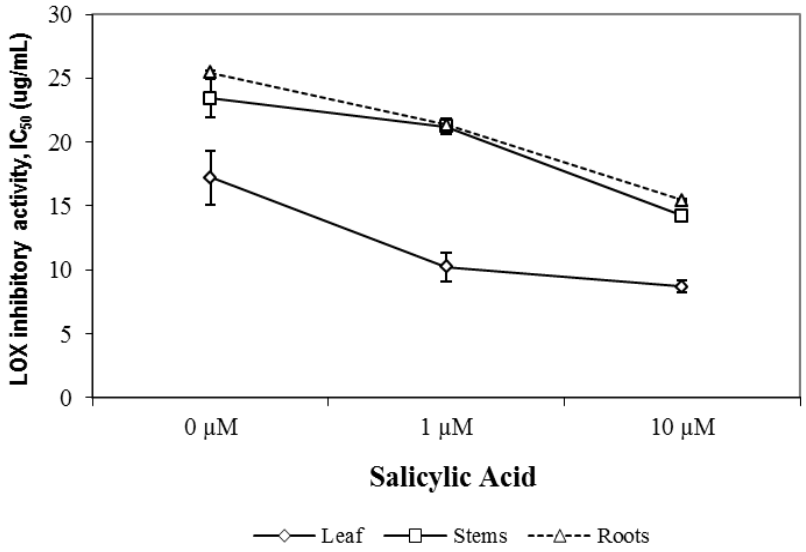

Fig. 4. Impact of salicylic acid on LOX inhibitory actvity of *Centella asiatica* expressed as IC_{50} (µg mL^{-1}). Nordihydroguaiaretic acid (NDGA) was used as a positive standard. The IC_{50} value for NDGA was 4.47. N= 15. Bars represent standard error of differences between means (SEM)

ability ($p< 0.05$) to inhibit LOX activity followed with 1 µM SA and 0 µM SA. The IC_{50} in the leaves was (8.71-17.21 µg/mL), stems (14.21 - 23.43 µg/mL) and in roots (15.43 - 25.45 µg/mL). It is important to note that *C. asiatica* plant extracts possessed significantly lower ($p< 0.05$) LOX inhibitory activity than that of a Nordihydroguaiaretic acid positive standard (IC_{50}= 4.47 µg/mL). Finally, LOX inhibition of *C. asiatica* extracts were higher than those for other common plants such as *Thespesia lampas* (600 µg/mL) [61]. The results reported here suggest that *C. asiatica* has potentially high anti-LOX effect, which might be related to the flavonoid content and antioxidant property of the plant extract (GSH and GSSG). This indicates, that *C. asiatica* supplemented with SA can have high anti-cancer properties [62,63].

4. CONCLUSION

The application of SA can promote the increase in production of secondary metabolites. This was shown by an increase in production of total flavonoid and saponin content as the concentration increased from 1 – 10 µM. This was followed by concomitant increased in glutathione and oxidize gluthatione that indicate increase in antioxidative properties under application of Salicylic application. The increase in photosynthetic capacity under application of SA might possibly enhance the production of SA that been justified by increased in CHS and PAL activity. Application of SA also was found to enhance LOX inhibitory activity, thus increase the anti-cancer properties of this plant.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Flora SJS, Gupta R. Beneficial effects of *Centella asiatica* aqueous extract against arsenic-induced oxidative stress and essential metal status in rats. Phytother Res. 2007;21:980–88.
2. Pittella F, Dutra RC, Junior DD, Lopes MTP, Barbosa NR. Antioxidant and cytotoxic activities of *Centella asiatica* (L) Urb. Int J Mol Sci. 2009;10;3713–721.
3. Li H, Gong X, Zhang L, Zhang Z, Luo F. Zhou Q. Madecassoside attenuates inflammatory response on collagen-induced arthritis in DBA/1 mice. Phytomed. 2009;16:538–46.
4. Shinomol GK, Muralidhara M. Prophylactic neuroprotective property of *Centella asiatica* against 3-nitropropionic acid induced oxidative stress and mitochondrial dysfunctions in brain regions of prepubertal mice. Neurotoxicology. 2008;29:948–57.
5. Dhanasekaran M, Holcomb LA, Hitt, AR, Tharakan B, Porter JW, Young KA. *Centella asiatica* extract selectively decreases amyloid beta levels in hippocampus of Alzheimer's disease animal model. Phytother Res. 2009;23:14–9.
6. Haleagrahara N, Ponnusamy K. Neuroprotective effect of *Centella asiatica* extract (CAE) on experimentally induced Parkinsonism in aged Sprague-Dawley rats. J Toxicol Sci. 2010;35:41–7.
7. Bian GX, Li GG, Yang Y, Liu RT, Ren JP, Wen LQ. Madecassoside reduces ischemia-reperfusion injury on regional ischemia induced heart infarction in rat. Biol Pharm Bull. 2008;31:458–63.
8. Cao W, Li XQ, Zhang XN, Hou Y, Zeng AG, Xie YH. Madecassoside suppresses LPS-induced TNF production in cardiomyocytes through inhibition of ERK,p38 and NF- B activity. Int Immunopharmacol. 2010;10:723–29.
9. Jaafar HZE, Mohamed HNB, Rahmat A. Accumulation and partitioning of total phenols in two varieties of *Labisia pumila* Benth. under manipulation of greenhouse irradiance. Acta Hort. 2008;797:387–92.
10. Lee SC, Norliza AL, Sze YL, Chew TL, Mohd RS, Ramlan AZ. Flavonols and phenolics from *Labisia pumila* (Kacip Fatimah). Food Chem. 2011;127:1186–192.
11. Stone BC. Notes on the genus *Labisia Lindyl* (Myrsinaceae). Malay Nat J. 1988;42:43–1.
12. Houghton PJ, Jamal JA, Milligan SR. Studies on *Labisia pumila* herb and its commercial products. J Pharm Pharmacol. 1999;51:236-39.
13. Zaizuhana S, Puteri JNMB, Noral'ashikin Y, Muhammad H, Rohana AB Zakiah, I. The in vivo rodent micronucleus assay of Kacip Fatimah (*Labisia pumila*) extract. Trop Biomed. 2006;23:214–19.
14. Choi HK, Kim DH, Kim JW, Ngadiran S, Sarmidi MR, Park CS. *Labisia pumila* extract protects skin cells from photoaging caused by UVB irradiation. J Biosci Bioeng. 2010;109:291–96.
15. Ibrahim MH, Jaafar HZE. The relationship of nitrogen and C/N ratio with secondary metabolites levels and antioxidant activities in three varieties of Malaysian Kacip Fatimah (Labisia pumila Blume). Molecules. 2011; 16: 5514–526.
16. Jamal JA, Houghton PJ, Milligan SR. Testing of *Labisia pumila* for oestrogenic activity using a recombinant yeast screen. J Pharm Pharmacol. 1998;50:79-2.
17. Shahidi F, Wanasundara PK. Phenolic antioxidants. Crit Rev Food Sci Nutr. 1992;32:67–103.
18. Ibrahim MH, Jaafar HZE, Rahmat A, Zaharah AR. The relationship between

phenolics and flavonoid production with total non structural carbohydrate and photosynthetic rate in *Labisia pumila* Benth. under high CO2 and nitrogen fertilization. Molecules. 2011;16:162–74.

19. Benhamou N. Elicitor-induced plant defence pathways. Trends Plant Sci. 1999;1:233–40.
20. Zhang C, Yan Q, Cheuk W, Wu J. Enhancement of tanshinone production in Salvia miltiorrhiza hairy root culture by Ag+ elicitation and nutrient feeding. Planta Med. 2004;70:147–51.
21. Zhao J, Davis LT, Verpoort R. Elicitor signal transduction leading to production of plant secondary metabolites. Biotechnol Adv. 2005;23:283–333.
22. Janas KM, Cvikrová M, Palagiewicz A, Szafranska K, Posmyk MM. Constitutive elevated accumulation of phenylpropanoids in soybean roots at low temperature. Plant Sci. 2002;163:369–73.
23. Zhao S, Zhang G, Liu D, Hu Z. Advances in research of pharmacological activities and biosynthesis pathway of water-soluble phenolic compounds of *Salvia miltiorrhiza*. Chin Trad Herb Drugs. 2004;35:341–44.
24. Chen JY, Wen PF, Kong WF, Pan QH, Zhan JC, Li JM, Wan SB, Huang WD. Effect of salicylic acid on phenylpropanoids and phenylalanine ammonia-lyase in harvested grape berries. Postharvest Biol Technol. 2006;40:64–2.
25. Sgarbi E, Fornasiero RB, Lins AP, Bonatti PM. Phenol metabolism is differentially affected by ozone in two cell lines from grape (*Vitis vinifera* L.) leaf. Plant Sci. 2003;165:951–57.
26. Solecka D, Kacperska A. Phenylpropanoid deficiency affects the course of plant acclimation to cold. Physiol Plantarum. 2003;119:253–62.
27. Kumaraswamy MV, Satish S. Antioxidant and anti-lipoxygenase activity of *Thespesia lampas* Dalz and Gibs. Advan Biol Res. 2008;2:56–9.
28. Chen Z, Silva S, Klessig D. Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. Science. 1993;262:1883-886.
29. Ibrahim MH, Jaafar HZE. Photosynthetic capacity, photochemical efficiency and chlorophyll content of three varieties of *Labisia pumila* benth. exposed to open field and greenhouse growing conditions. Acta Physiol Plant. 2011;33:2179–185.
30. Ibrahim MH, Jaafar HZE, Asmah R, Zaharah AR. Involvement of Nitrogen on flavonoids, glutathione, anthocyanin, ascorbic acid and antioxidant activities of Malaysian medicinal plant Labisia pumila Blume (Kacip Fatimah). Int J Mol Sci. 2012;13:393–08.
31. Makkar HPS, Siddhuraju S, Siddhuraju P, Becker K. Plant Secondary Metabolites; Humana Press: Totowa, NJ, USA; 2007.
32. Martinez-Tellez MA, Lafuente MT. Effects of high temperature conditioning on ethylene, phenylalanine ammonia lyase, peroxidase and polyphenol oxidase in flavedo of chilled “Fortune” mandarin fruit. J Plant Physiol. 1997;150:674–78.
33. Obinata N, Yamakawat T, Takamiya M, Tanaka N, Ishimaru K, Kodama T. Effects of salicylic acid on the production of procyanidin and anthocyanin in cultured grape cell. Plant Biotechnol. 2003;20:105–11.
34. Castillo FJ, Greppin H. Extracellular ascorbic acid and enzyme activities related to ascorbic acid metabolism in Sedum Album L. leaves after ozone exposure. Env Exp Bot. 1988;28:232-33.
35. Wu H. Affecting the activity of soybean lipoxygenase-1. J. Mol. Graph. 1996;14: 331–37.
36. Ibrahim MH, Jaafar HZE. Photosynthetic capacity, photochemical efficiency and chlorophyll content of three varieties of *Labisia pumila* Benth. exposed to open field and greenhouse growing conditions. Acta Physiol Plant. 2011;33:2179–185.
37. Mutlu S, Atici Ö, Nalbantoglu B. Effects of salicylic acid and salinity on apoplastic antioxidant enzymes in two wheat cultivars differing in salt tolerance. Biol Plantarum. 2009;53:334–38.
38. Fariduddin Q, Hayat S, Ahmad A. Salicylic acid influences net photosynthetic rate, carboxylation efficiency, nitrate reductase activity, and seed yield in *Brassica juncea*. Photosynthetica. 2003;41:281-84.
39. Alexander K, Rusina Y, Tibor J, Gabriella S, Losanka P. Treatment with salicylic acid decreases the effect of cadmium on photosynthesis in maize plants. J Plant Physiol. 2008;165:920-31.
40. Liu C, Zhan J, Yuan YB, Yu C,Yu–Long F, 1999. Effects of salicylic acid on the

photosynthesis of apple leaves. Acta Hort Sinica. 1999;26:261–62.

41. Ghasemzadeh A, Jaafar HZE. Effect of salicylic acid application on biochemical changes in ginger (*Zingiber officinale* Roscoe). J Med Plant Res. 2012;6:790-95.
42. Shi QH, Zhu ZJ. Effects of exogenous salicylic acid on manganese toxicity, element contents and antioxidative system in cucumber. Environ Exp Bot. 2008;63: 317–26.
43. Okem A, Stirk WA, Street RA, Finnie JF, Van Staden J. Effects of Cd and Al stress on secondary metabolites, antioxidant and antibacterial activity of Hypoxis hemerocallidea Fisch. & C.A. Mey. Plant Physiology Biochem. 2015;97:147-55.
44. Okem A, Southway C, Stirk WA, Finnie JF, Van Staden J. Effect of cadmium and aluminum on growth, metabolite content and biological activity in *Drimia elata* (Jacq.) Hyacinthaceae. South African J Bot. 2015;98:142-47.
45. Thiago AN, Giuliana S, Camilo EV, Carla B, Robson C, Marcelo E, Andrea G. Methyl jasmonate and salicylic acid are able to modify cell wall but only salicylic acid alters biomass digestibility in the model grass. Plant Sci. 2017;263:46-4.
46. Abdulaziz MA, Alrashdi A, Mohamed AA, Saleh AM, Awad AA. Quality, antioxidant compounds, antioxidant capacity and enzymes activity of 'El-Bayadi' table grapes at harvest as affected by preharvest salicylic acid and gibberellic acid spray Sci Hort. 2017;220:243-49.
47. Tolonen M, Taipale M, Viander B, Pihlava JM, Korhonen H, Ryhänen EL. Plant-derived biomolecules in fermented cabbage. J Agric Food Chem. 2002; 50: 6798-803. Vadim D. Mechanisms of oxidative stress in plants: From classical chemistry to cell biology, Environ Exp Bot. 2015;109:212-28.
48. Gomes AM, Soares AM, Garcia QS. Phosphorous and sulfur nutrition modulate antioxidant defenses in plants exposed to arsenic. J Hazardous Mat. 2014;276:97-104.
49. Gérard A, Paul B, Sylvain R, Maurice S. Theoretical investigations on the conversions of cyclic polysulfides to acyclic polysulfide diradicals and subsequent reactions of biological interest. Tetrahedron. 2017;73:3492-496.
50. Sabrina I, Melina T, Ornella O, Jachym S, María I, Tomas M, Elizabeth A. Transgenic plants and hairy roots: Exploiting the potential of plant species to remediate contaminants, New Biotech. 2016;33:625-635.
51. Thiago Al, Giuliana S, Camilo E, Carla B, Robson C, Marcelo E, Andrea G. Methyl jasmonate and salicylic acid are able to modify cell wall but only salicylic acid alters biomass digestibility in the model grass. Plant Sci. 2017;263:46-4.
52. Dalton DA, Russell SA, Hanus FJ, Pascoe GA, Evans HJ. Enzymatic reactions of ascorbate and glutathione that prevent peroxide damage in soybean root nodules. Proc Natl Acad Sci. 1986;83:3811-813.
53. Fajer ED, Bowers MD, Bazaaz FA. The effects of nutrients and enriched CO2 environment on the production of carbon based allelochemicals in Plantago: A test of the carbon/nutrient balance hypothesis. Am Nat. 1992;140:707–723.
54. Campos AD, Ferreira AG, Hampe MMV, Antunes IF, Brancão N, Silveira EP, da Silva, JB, Osório VA. Induction of chalcone synthase and phenylalanine ammonia-lyase by salicylic acid and *Colletotrichum lindemuthianumin* common bean Determination of Antioxidant Activities. Braz J Plant Physiol. 2003;15:129–34.
55. Ozeki Y, Komamine A, Noguchi H, Sankawa U. Changes in activities of enzymes involved in flavonoid metabolism during the initiation and suppression of anthocyanin synthesis in carrot suspension cultures regulated by 2,4-dichlorophenoxyacetic acid. Physiol Plant. 2006;69:123–28.
56. Winkel-Shirley B. Flavonoid Biosynthesis. A Colorful Model for Genetics, Biochemistry, Cell Biology, and Biotechnology. Plant Physiol. 2001;126: 485–93.
57. Shim IS, Momose Y, Yamamoto A, Kim DW, Usui K. Inhibition of catalase activity by oxidative stress and its relationship to salicylic acid accumulation in plants. Plant Growth Regul. 2003;39:285–92.
58. Thulke O, Conrath U. Salicylic acid has a dual role in the activation of defence related genes in parsley. Plant J. 1998;14: 35–2.
59. Lafuente MT, Sala JM, Zacarias L. Active oxygen detoxifying enzymes and

phenylalanine ammonia-lyase in the ethylene-induced chilling tolerance in citrus fruit. J Agri Food Chem. 2004;52:3606–611.

60. Chandra A, Anand A, Dubey A, Effect of salicylic acid on morphological and biochemical attributes in cowpea. J Environ Biol. 2007;28:193-96.

61. Moreno JJ. New aspects of the role of hydroxyeicosatetraenoic acids in cell growth and cancer development. Biochem Pharmacol. 2009;77:1–10.

62. Kumaraswamy MV, Satish S. Antioxidant and anti-lipoxygenase activity of Thespesia lampas Dalz and Gibs. Advan Biol Res. 2008;2:56–9.

63. Dobrian AD, Lieb DC, Cole BK, Taylor-Fishwick DA, Chakrabarti SK, Nadler JL. Functional and pathological roles of the 12- and 15-lipoxygenases. Prog Lipid Res. 2011;50:115–31.

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