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*Full Length Research Paper*

# Influence of witches' broom disease on catechin, epicatechin and their enantiomers concentrations in *Citrus aurantifolia*

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**Recently, witches' broom disease causes a large problem for *Citrus aurantifolia* in a few countries such as Oman and Iran. The results show that the metabolism of plant cells are greatly changed due to biotic stresses leading plant to an optimized metabolic response for defense. Herein, we showed that the infection of *Citrus aurantifolia* to Witches' broom disease has a close relation with the content of catechin and epicatechin metabolites which are well known for their biological properties. These changes in the metabolites levels were in proper agreement with the activities of phenylalanine ammonia-lyase(PAL) and chalcone synthase (CHS) as the key enzymes of the biosynthesis of catechin and epicatechin. Infection of the plant resulted in the higher levels of both the metabolites with up regulated activities of PAL and CHS. Enantiomeric analysis of the metabolites revealed that infection of the *Citrus aurantifolia* cause to change the enantiomeric composition of catechin and caused accumulation of the (+)-enantiomer, which is a known antibacterial agent compared to (-)-catechin. Results of this study showed that plant is able to regulate the chirality of necessary metabolites when acquired in the stress conditions.**

**Key words:** *Citrus aurantifolia*, witches' broom disease, catechin, epicatechin, PAL, CHS, enantiomers.

## INTRODUCTION

The biological distortion caused by witches broom disease on *Citrus aurantifolia* does influence the agricultural economy and related industries of countries which produce this fruit. Usually, the determination of pathway of this biological distortion is essential to find biomarkers to diagnose the disease and prevent its dispersion. Catechin and epicatechin are epimers belonged to the class of flavan 3-ols which have isolated from a variety of natural sources(Tsanova-Savova et al., 2005; Maoela et al., 2009). It seems that a pathogenic infection and stresses induces accumulation of phenolic compounds as a consequence of defense reaction in different plant tissues (Mazid et al., 2011; Qawasmeh et al., 2012). The research was performed on healthy green walnut fruits and on fruits infected with *Xanthomonas arboricola* pv. *Juglandis* and the results showed that in comparison to healthy husk tissue, the infected husks contained up to 23 fold more catechin (Mikulic-Petkovsek et al., 2011). Infection of pear leaves by *Gymnosporangium sabincae* increased 6-fold in extractable flavon-3-ols from the boundary zones in comparison with non-infected tissues (Treutter and Feucht, 1990). Concentration of catechin in cotton seedling hypocotyls increased with age and resulted in more resistance to sore shin disease which is incited by *Rhizoctonia solani* (Hunter, 1987). Studies on the infected cherry leaves provided evidence that the injured leaves had higher concentration of catechin and epicatechin (Nahrstedt et al., 1987).

Moreover, catechin and epicatechin have two chiral centers, so they are four stereoisomers but (-)-epicatechin and (+)-catechin are the most optical isomers found in nature compared to the other enantiomers which were seldom found (Nahrstedt et al.,1987). The important question is what happens to the enantiomeric composition

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of catechin after infections? Some biotic and abiotic stresses such as disease, temperature and pH could change the ratio of enantiomers of catechin (Ghassempour et al., 2011; Kofink et al., 2007). Also, natural and synthetic compounds influence the enantiomeric ratio of natural compounds (Orhan, 2012). Nornicotine is synthesized by demethylation of nicotine in leaf and root (*Nicotiana tabacum* L.), as major metabolite. Recent reports showed that the major nicotine demethylase CYP82E4 activity in tobacco demonstrated that CYP82E4 selective demethylated (S)-nicotine and obtain different enantiomeric composition of nornicotine in tobacco (Lewis et al., 2010; Cai et al., 2012).

Ghassempour et al. showed that when wheat infected by *puccinia triticia*, not only the content of catechin and epicatechin were increased, but also the ratio of catechin**'** enantiomer did change (Ghassempour et al., 2011). Other studies performed by Ritter et al. showed that the rate of absorption of enantiomers by the natural organisms were different, in a way that (+)-catechin was more absorbed than (-)-catechin(Ritter et al., 2010). Under the influence of high temperature (-)-epicatechin was converted into (-)-catechin, indicating an epimerization reaction. To confirm the influence of heat treatment during the roasting process, Kofink et al. roasted unfermented cocoa beans which only contain (-)-epicatechin and (+)-catechin and undetectable levels of (-)-catechin(Kofink et al., 2007).

The enzyme phenylalanine ammonia-lyase (PAL) catalyzes deamination reaction of the amino acid phenylalanine at the gateway from the primary metabolism into the important secondary phenylpropanoid/phenolic metabolism in plants. CHS condenses three malonyl-CoA molecules with cinnamoyl-CoA to produce chalcone. Effects of different stresses on PAL and CHS activities have been previously reported and are considered an excellent marker of plant disease resistance against pathogens (Singh et al., 2012; Yonghong et al., 2013; Campos et al., 2003).

In this paper, the effect of witches' broom disease on the content of catechin and epicatechin and its ratio of enantiomers as biomarkers in *Citrus aurantifolia* along with changes in the activities of PAL and CHS is investigated.

## MATERIAL AND METHODS

### Plant material and inoculation

Twenty healthy 1-year-old *Citrus aurantifolia* trees grown in the greenhouse were arranged on the greenhouse bench. Specimens from *Citrus aurantifolia* trees infected with witches' broom disease were grafted to half of them randomly and were covered for 45 days with plastic bags to increase humidity. All trees were kept under natural light conditions at a temperature of 25-28°C. After six months, all trees successfully grafted with scions of diseased lime became infected. Randomly, five infected trees, as a treatment, and five healthy trees, as a control, were sampled and used for analysis. The standard chemicals were purchased from Merck (Darmstadt, Germany). HPLC grade solvents were purchased from Merck. MilliQ-water was prepared by a MilliQ-System (Mil-lipore, Saint-Quentin-en-Yvelines, Fran

The instruments used in this study were as follows: a LC-10AD VP liquid chromatography pump (Shimadzu Co., Japan), a SPD-10A UV–VIS detector, a computer-controlled system with GC_CHROM software and 20 µL sample loop. The analytical HPLC columns were ODS-120A Varian column (250 mm × 4.6 mm, 5 µm) and Eurecel 01 column (120 mm × 8 mm, 5 µm) packed with cellulose (tris 3,5-dimethylphenylcarbamate (Knauer, Germany).

Thermo Fisher LCQ ion trap mass spectrometer (Bremen, Germany) with the ability of scanning the range of 10-2000 m/z was used for the analysis of the samples. Also, software of X-calibur was utilized. Negative mode of ESI-MS under capillary voltage of -2.0 KV and skimmer cone voltage of +20 V was applied to determine the molecular weights of the sample components.

The dried and powdered leaves of *Citrus aurantifolia* (0.4 g) were extracted by ethanol, under continuous stirring at 300 × g, during 2 hour. After filtration, the extraction solvent was diluted with water (1:1 v/v). The removal of less polar compounds was extracted with chloroform. Then the aqueous phase was collected and the impurities associated with the chloroform phase were discarded. A second partition with water/ethyl acetate (1:1 v/v) was applied. Catechin and epicatechin were extracted by ethyl acetate which was then injected to HPLC(Row and Jin, 2006).

To separate and identify catechin and epicatechin in sample reversed phase HPLC was used. An ODS-120A Varian column (250 mm × 4.6 mm i.d., 5 µm) was connected to the HPLC system (Shimadzu, Kyoto, Japan). 20 µl of the each sample was injected and eluted with methanol and water (20:80v/v) at a flow rate of 1 ml/min and the UV-Vis detector was set at 280 nm. The ability of catechin and epicatechin separations was evaluated by the analysis of spiked crude extract with standard solution of catechin and epicatechin using the optimum chromatographic separation conditions.

After collection of catechin and epicatechin from end of C18 column (section 2.5) solvent was evaporated and the extracted sample was dissolved in ethanol. The column used for these analysis was Eurecel 01 column (120 mm × 8 mm, 5 µm) by using n-hexane: ethanol: acetic acid (64.5:35:0.5 v/v/v) as a mobile phase at a flow rate: 1 ml/min and temperature 40ºC. Ito et al.( 2003).

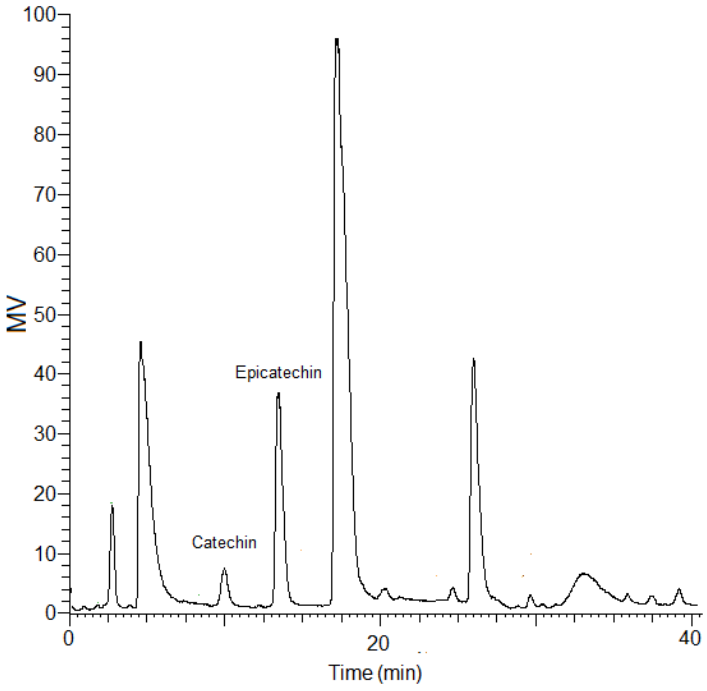
One gram of each plant was homogenized in 5ml of phosphate buffer (pH 7.2) at 4$^{o}$C and were centrifuged at 10,000 × g for 15 min at 4$^{o}$C. The supernatant was diluted

**Table 1.** comparison results of the *Citrus aurantifolia* traits as affected by witches' broom disease[a].

| | Catechin | nihcetacipE | PAL |
|---|---|---|---|
| Healthy *Citrus aurantifolia* | 21.46±0.99 | 51.72±0.49 | 4.560±0.276 |
| Infected *Citrus aurantifolia* | 62.53±1.08 | 113.74±7.39 | 10.216±0.311 |
| LSD (0.05) | 4.084 | 20.588 | 1.156 |
| Significance | ***[b] | | |

[a]Catechin and epicatechin: µg per (g FW); PAL activity: nmolcinnamic acid per (mg protein) $h^{-1}$
[b]Levels of significance are represented by at *P<0.05, **P<0.01 and ***P<0.001.

**Figure 1**. Determination of catechin and epicatechin by HPLC-UV on C18 column in leaves of *Citrus aurantifolia.*




5 times and then was used as enzyme source (Havir and Hanson, 1970; Hodgins, 197

The PAL activity was expressed as one unit of PAL which deaminate 1.0 µmole of L-phenylalanine to trans- absorbance was measured at 270nm. This reaction was as test sample. Blank was prepared with same composition only 0.1 ml 150 mMTrisHCl buffer, pH 8.5 was used instead of enzyme solution(Havir and Hanson, 1970; Hodgins, 1971).

0.15 ml of enzyme solution was incubated with 0.05 ml Malonyl-CoA (2.5 n mole) and 0.05 ml 4-Coumaroyl-CoA (1 nmole) (prepared in 100 mMTrisHCl buffer, pH 8.5 at 30°C) and 0.75 ml 100 mMTrisHCl buffer at 37°C for 1.5 h. Then, the reaction was stopped by adding 100 µl 6N cinnamate and NH3 per minute at pH 8.5 at 30°C. Determination of PAL activity was done as follows: 0.1 ml of enzyme solution was incubated with 2 ml of 3 mM L-phenylalanine solution and 0.9 ml of water and HCl. CHS activity was measured at 300 nmand expressed as p molenaringenin $h^{-1}$ mg $protein^{-1}$. Blank was prepared with same composition only 0.15 ml 150 mMTrisHCl buffer, pH 8.5 was used instead of enzyme solution(Spribille andForkmann, 1982).

## Statistical analysis

The experiment was carried out by completely random design

(CRD) which each sample consisted of five replicates. The means were separated by least significant difference (LSD) test andall data in the Tables 1 represent the means ± SE of five replications.The data were statically analyzed by Statistical Analysis System (SAS) software.

## RESULTS

The influence of witches' broom disease on the concentration of catechin and epicatechin in leaves of *Citrus aurantifolia* has been investigated. Catechin and epicatechin were extracted from healthy and infected leaves of *Citrus aurantifolia* and analyzed by HPLC (Figure 1) at optimal obtaining extraction and chromatographic conditions. Also, HPLC condition of catechin and epicatechin permitted to collect them at the end of HPLC column and analyzed for their enantiomers contents by chiral column.The analysis of variance (ANOVA) and mean comparison results of witches' broom disease on catechin and epicatechin in *Citrus aurantifolia* have showed in Table 1. Data presented in Table 1 show that witches' broom disease have significantly increased amount of catechin and epicatechin as 191 and 119 % respectively, compared with the healthy *Citrus aurantifolia.*

So, the observation of these enzymes activity have been performed in healthy *Citrus aurantifolia* and infected *Citrus aurantifolia* to witches' broom disease. The results showed that PAL and CHS activity significantly increased in *Citrus aurantifolia* infected to witches' broom diseasein comparison with healthy leaves of *Citrus aurantifolia* from 4.560 to 10.216 units and 56.048 to 109.112 units, respectively (Table 1).

After collection of catechin and epicatechin collected by reversed phase HPLC using C18 column, the enantiomeric separation of these metabolites was performed with normal phase HPLC using cellulose tris(3,5-dimethylphenyl carbamate) column. The results showed that the catechin contents in the leaves of *Citrus aurantifolia* were 61% for (+)-catechin and 39% for (-)-catechin (Table 2). The same ratio in infected *Citrus aurantifolia* was 79:21 (Table 2).

## DISCUSSION

We know that phenolic compounds were produced as a consequence of defense reaction. So after analysis of catechin and epicatechin by HPLC, confirming of these compounds was done by retention time and Mass spectroscopy. In HPLC chromatogram, peaks of catechin and epicatechin were observed at the retention times of 8.0 and 12.5 min, respectively. For confirmation of catechin and epicatechin peaks, negative mass spectrometry of these compounds was obtained by liquid chromatography-mass spectrometry (LC-MS) system. Catechin and epicatechin are stereoisomers thus they have same theoretical molecular weight at 290.26 g/mol. Negative mass spectra of these peaks (Figure 2) gave an experimental value of the parent ion at m/z 289.28, $(M-H)^-$ of catechin. Further mass analyze in MS/MS on 289.28 led to m/z 245.03, $(M-H-[C_2H_4O])^-$ and 205.15, $(M-H-[C_4H_4O_2])^-$ (Fig. 2) which were similar to results obtained by Kajdzanoska et al (2010).

According to this finding (Table 1), it can be speculated that infection of plants caused stimulation of the biosynthesis of catechin and epicatechin as a defense mechanism, probably to prevent more infection. It was found that catechin and epicatechin levels, which are normally produced through shikimate pathway, can be induced by stresses e.g. temperature, physical injury, diseases and elicitors (Ghassempour et al., 2011; Yamamoto et al., 2000). PAL and CHS are two key enzymes that are involved in phenylpropanoid biosynthesis. The first reaction of this biosynthetic pathway is the deamination of phenylalanine to cinnamic acid by PAL. In the next reaction of the metabolic pathway, CHS condenses three malonyl-CoA molecules with cinnamoyl-CoA to produce chalcone. This condensation is the main branch in the pathway for the production of flavonoids (Singh et al., 2009;Levine et al., 1994; Dixon et al., 1992).The activity of these enzymes increase in response to the change in the environmental conditions and also biotic and abiotic stresses (Levine et al., 1994; Reyes and Zevallos, 2003). Therefore, it is strongly suggested that these enzymes might be related to induction of catechin and epicatechinin *Citrus aurantifolia* suffering from disease. The increase activities of enzymes are compatible with increase of catechin and epicatechin concentration levels in *Citrus aurantifolia* infected to witches' broom disease samples. Thus concentration of catechin and epicatechin and also increase of activity of PAL and CHS are biomarkers of witches' broom disease. In another report, shotgun proteomic analysis of the mexican lime tree infected with "*Candidatus Phytoplasma aurantifolia*" showed a significant increase in proteins in healthy *Citrus aurantifolia* and infected *Citrus aurantifolia* (Monavarfeshani et al., 2013).

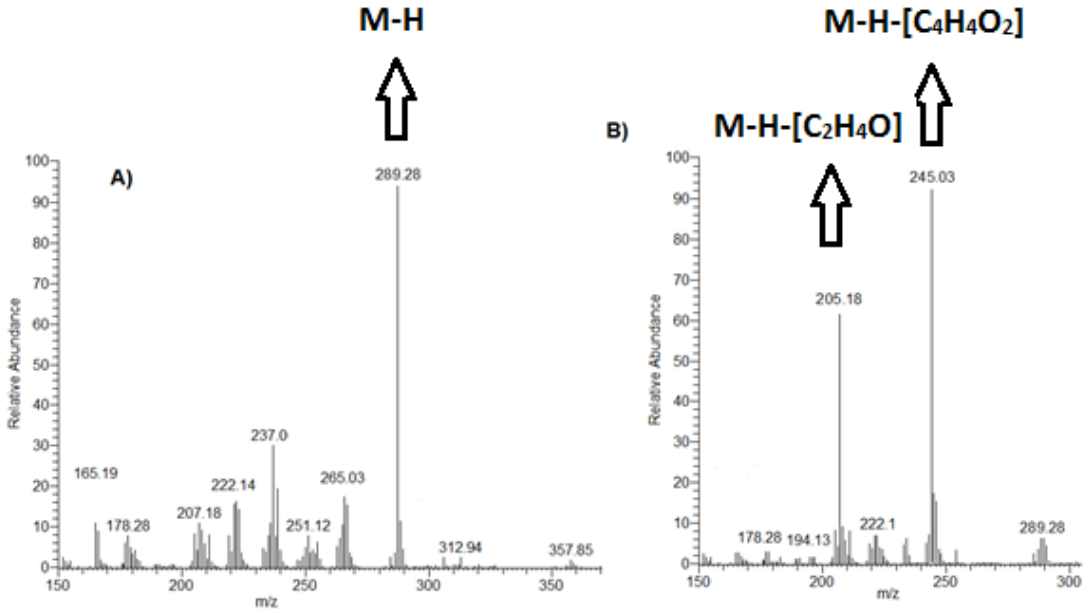
Can a uniq ue biomarker for *Citrus aurantifolia* infected with witches' broom disease be identified? Ghassempour et al. investigated the impact of disease on enantiomeric composition of catechin and epicatechin (Ghassempour et al., 2011).Thus the impact of witches' broom disease on *Citrus aurantifolia* not only changed catechin concentration and activity of PAL and CHS enzymes, but significantly it influenced the ratio of enantiomers. Study on enantiomeric ratio of epicatechin showed that only (-)-epicatechin was produced in all samples, either healthy or infected. The main focus on the catalytic mechanism of ammonia lyases enzymes, such as PAL and/or the influence of the enzymes as catalysts for the synthesis of enantiomers was reported (Villiers et al., 2012). Thus,

**Table 2.** Enantiomeric ratio of catechin in healthy and infected *Citrus aurantifolia.*

| | (+)-Catechin (%) | (-)-Catechin (%) |
|---|---|---|
| Healthy *Citrus aurantifolia* | 61 | 39 |
| Infected *Citrus aurantifolia* | 79 | 21 |
| Significance | *[a] | * |

aLevels of significance are represented by at *P<0.05, **P<0.01 and ***P<0.001.

**Figure 2.** A) Negative ESI-MS spectrum of catechin, B) MS-MS spectrum of m/z=289.28.




the increase of PAL and CHS activities in *Citrus aurantifolia* infected to witches' broom disease samples could influence on enantiomeric ratio of catechin and epicatechin.

## CONCLUSIONS

Results of this study showed that infection of the plant *Citrus aurantifolia* by witches' broom disease has a close relation to the level of catechin and epicatechin in the leaves. This finding reveals that infection of the plant results in the higher production and accumulation of catechin and epicatechin in the cells which is in agreement with PAL and CHS activities. Also change in the metabolism of the plant due to this infection has a high impact on the enatiomeric composition of catechin, directing the cells to accumulate (+)-ennatiomer which is a well- known antibacterial agent.

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