Proceedings of the 7th Congress on Plant Protection

Доклады 7-ого Конгресса по защите растений

Plant Protection Society of Serbia
Общество по защите растений Сербии

International Organization for Biological Control
-East Palearctic Regional Section (IOBC-EPRS)
-West Palearctic Regional Section (IOBC-WPRS)

Международная организация по биологической борьбе
- Восточно палеарктическая региональная секция (МОББ-ВПРС)
- Западно палеарктическая региональная секция (МОББ-ЗПРС)

Editors/Редакторы

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Proceedings of the 7th Congress on Plant Protection

„Integrated Plant Protection – a Knowledge-Based Step towards Sustainable Agriculture, Forestry and Landscape Architecture“

(November 24-28, 2014, Zlatibor, Serbia)

Доклады 7-ого Конгресса по защите растений

„Интегрированная защита растений - научно обоснованный шаг к устойчивому развитию сельского хозяйства, лесоводства и пейзажной архитектуры“

(24-28 ноября 2014 года, Златибор, Сербия)

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on the occasion of the 60th anniversary of the PPSS
по поводу 60-летия ОЗРС

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PREFACE

The Plant Protection Society of Serbia (PPSS) and two regional sections of the International Organization for Biological and Integrated Control (IOBC-EPRS and IOBC-WPRS), on the occasion of the 60th anniversary of the PPSS organized VII Congress on Plant Protection with a motto: *"Integrated Plant Protection – a Knowledge-Based Step towards Sustainable Agriculture, Forestry and Landscape Architecture"* (November 24-28, 2014, Zlatibor, Serbia). The Congress enabled exchange of up-to-date scientific and technical information on plant protection in Agriculture, Forestry and Landscaping among researchers, teachers, experts in extension and public services and the business community, and promoted international cooperation. The Congress focused on basic knowledge and management practices established in plant protection, as well as on the development of alternative and innovative approaches. In addition, biological control as an important tool for the control of the harmful organisms with a minimal risk for ecosystems was discussed. A total of 209 contributions was presented - 8 keynote presentations, 28 oral presentations and 173 poster presentations - prepared by 467 authors from 26 countries. The Congress Proceedings comprise 65 contributions - 5 keynote presentations and 60 oral and poster presentations in six sessions, prepared by the authors from 18 countries (Algeria, Austria, Bosnia-Herzegovina, France, Georgia, Hungary, Italy, Kazakhstan, Montenegro, Poland, Russia, Rwanda, Serbia, Slovenia, Switzerland, Turkey, Uganda, USA). All contributions were reviewed by members of the Scientific Committee and other reviewers selected and invited by the editors of this publication.

Belgrade, November 2015 Editors

ПРЕДИСЛОВИЕ

Общество по защите растений Сербии (ОЗРС), Международная организация по биологической борьбе с вредными животными и растениями - Восточно палеарктическая региональная секция (МОББ-ВПРС) и Международная организациая по биологической борьбе и интегрированной системе защиты растений - Западно-палеарктическая региональная секция (МОББ-ЗПРС), по поводу 60-летия ОЗРС организировали VII Конгресс по защите растений, под девизом: *"Интегрированная защита растений - научно обоснованный шаг к устойчивому развитию сельского хозяйства, лесоводства и пейзажной архитектуры"* (24-28 ноября 2014 года, Златибор, Сербия). Цель Конгресса была обеспечение континуитета взаимообмена научно-техническими информациями, отвечающими современным требованиям защиты растений в сельском хозяйстве, лесоводстве и пейзажной архитектуре, которые представляют интерес для ученых, исследователей, преподавателей, экспертов-советников в области сельского хозяйства, лесоводства и пейзажной архитектуры, специалистов государственных и коммунальных служб, деловых кругов и средств массовой информации. Целью Конгресса является и продолжение содействия развитию и популяризации международного сотрудничества. Конгресс был концентрирован на основные знания и практический менаджмент в защите растений, а также на развитие алтернативних и новых подходов. Биологическая защита каторая представляет значительный способ для безопасной борьбы с вредними организмими была тоже рассмотривана. На конгрессе представлено 209 презентаций - 8 докладов по приглашению, 28 устных и 173 постер презентаций - которые подготовило 467 авторов из 26 стран. Сборник имеет 65 докладов - 5 докладов по приглашению и 60 устных и постер презентаций, распределенных в шести секциях. Авторы докладов приехали из 18 стран (Алжир, Австрия, Босния-Герцеговина, Франция, Грузия, Венгрия, Италия, Казахстан, Черногория, Польша, Россия, Руанда, Сербия, Словения, Швейцария, Турция, Уганда, США). Рецензенты всех опубликованных докладов в сборнике – члены Научного совета и другие рецензенты, выбранные редакторам этого издания.

Белград, Ноября 2015 Редакторы

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DETERMINATION OF PHENOLIC COMPOUNDS IN PLANT EXTRACTS BY HPLC-DAD

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ABSTRACT

Plants have the ability to synthesize secondary metabolites, biologically active substances involved in defense mechanisms against insects, pathogenic fungy and bacteria. Biochemical bases of their activity are related to the presence of specific molecules, among others, phenolic compounds. The aim of this study was to develop the validated method for the determination and quantification of phenolic acids, kaempferol and quercetin in ethanol leaf extracts of *Morus alba* L. and *Halascya sendtneri* (Boiss.) and leaf and bark extract of *Ailanthus altissima* (Mill.). The separation, quantification and validation of the individual phenols were performed by high performance liquid chromatography with diode-array detection (HPLC-DAD). The HPLC-DAD separation was achieved using a ZORBAX SB-Aq (5 µm particle size: 4.6 x 250 mm, Agilent). The mobile phase was acetonitrile with 2.0% acetic acid and Milli-Q water with 2.0% acetic acid in gradient mode, with the flow rate 1.0 ml/min. The obtained LOQs for all investigated phenolic acids were 0.03 µg/ml. The precision values, expressed as relative standard deviation (RSD, %), were lower than 10.19%. The developed HPLC-DAD chromatographic procedure exhibits linearity (R^2>0.99) for the concentrations from 10.0 to 100.0 µg/ml with the repeatability RSD less than 12.00%. An efficient, sensitive and reliable method is developed which can be applied in the analysis of real plant material samples to ferulic, trans-cinnamic, 2-hydroxy cinamic, gallic, caffeic, p-coumaric and chlorogenic acid, kaempferol and quercetin.

Key words: phenolic compounds, plant extracts, HPLC-DAD

INTRODUCTION

Current limitations in chemical pest control methods specify the scope for identifying safe, non-polluting rational methods in the control of economically important agricultural pests. Plants have developed many chemical defense mechanisms against insects in the evolution process and the ability to synthesize a broad range of different volatile and non-volatile chemical compounds called secondary metabolites (Wink 1993; Howe and Jander 2008), such as alkaloids, polyphenols, terpenoids, steroids, essential oils, lignans, sugars, and fatty acids, (Regnault-Roger et al. 2004; Isman 2006, Erturk et al., 2006; Shields et al., 2006; Koul, 2008). Naturally occurring plant compounds can affect the physiology or modify behavior of insect herbivores in terms of feeding and these are potentially suitable for use in integrated pest management (Schmutter, 1992).

The role of phenols in chemoecology especially on feeding behavior of herbivorous insects has been recognized since 1959, when Fraencke described phenolic compounds as "trigger" substances which induce or prevent the uptake of nutrients by animal herbivores. Plant polyphenols are secondary metabolites that constitute one of the most common and widespread groups of natural products (Cheynier et al., 2012). According to Bandaranayake (2002) these plant compounds have toxicological, pharmacological and ecological importance. Harborne (2002) emphasized that they have been implicated in diverse functional roles, including plant resistance against microbial pathogens and animal herbivores (insects), protection against solar radiation, besides reproduction, nutrition and growth.

High-performance liquid chromatography with diode array detection method (HPLC-DAD) is a widely used method for the analyses of phenolic compounds (Irakli et al., 2012; Andrejev and Bursić, 2013; Zhang et al., 2013; Bursić et al., 2013, Šućur et al., 2014). Although, in a recent literature data the liquid chromatography with tandem mass spectrometry (LC–MS/MS) systems is use for the determination of these substances (Gomez-Caravaca et al., 2013; Bursić et al., 2014).

The aim of this research was to develop and validate the HPLC-DAD method for the detection of phenolic compounds, such as ferulic, trans-cinnamic, 2-hydroxy cinamic, gallic, caffeic, p-coumaric and chlorogenic acid, quercetin and kaempferol in plant extracts. The method was evaluated in terms of linearity (R^2), reproducibility, limits of detection (LODs) and limits of quantification (LOQs).

MATERIALS AND METHODS

Chemicals and apparatus

All solvents used were of chromatography grade and were obtained from J.T. Baker (Deventer, Netherlands). The analytical standards manufactured by Sigma-Aldrich, which were used in the research work are ferulic acid (99.0%), trans-cinnamic acid (99.0%), 2-hydroxy cinamic acid (97.0%), gallic acid (99.9%), caffeic acid (98.0%), p-coumaric acid (98.0%), chlorogenic acid (95.0%), quercetin (98.0%), kaempferol (97.0%). The stock standard solutions were prepared by dissolving an analytical standard in methanol while the working solution i.e. the mixture of the studied phenol compounds was obtained by mixing and diluting the stock standards with mobile phase resulting in the final mass concentration of 100 mg/ml. The composite mixtures of all phenol compounds at appropriate concentrations were used to spike samples in validation data settings. Acetic acid was of "pure for analysis" grade by Carl Roth.

HPLC analysis

The chromatographic separation for phenolic compounds was achieved using the Agilent 1100 (Agilent Technologies, USA) HPLC system with a binary pump and diode array detector - DAD. The phenolic acids were separated on a ZORBAX SB-Aq (5 μm particle size: 4.6 x 250 mm, Agilent) column. The extracts were filtered through 0.45-μm syringe filters and directly injected through a 30 μl fixed loop into the column.

The mobile phase was acetonitrile with 2.0% acetic acid (solvent A) and Milli-Q water with 2.0% acetic acid (solvent B) in gradient mode, with the flow rate of 1.0 ml/min. This was equipped with a ZORBAX SB-Aq column. The gradient was as follows: 92% A at 0 min, 80% A at 18 min, 60% A at 25 min, 55% A at 30 min, 35% A at 40 min and 20% A at 42 min. Stop time was 2.5 minutes.

Validation parameters

The detector linearity response was checked by preparing the blank plant extract sample (leaves and root, separately) according to the Generalić et al. (2012) method and after the extraction the residue was diluted in 1.5 ml of the phenol compounds mixture standard in mass concentrations of 10.0, 25.0, 50.0 and 100.0 μg/ml.

The extract preparation

Plant material (10.0 g) was extracted with 70% ethanol (100.0 ml) as a solvent. Ethanol extracts of *Morus alba* L. and *Halascya sendtneri* (Boiss.) leaves and *Ailanthus altissima* (Mill.) leaves and bark were used in the analysis. The extracts were filtered through 0.45 μm syringe filters and directly injected into the HPLC-DAD. The repeatability of the method was determined by analyzing the sample of the same mass concentration level (10.0 μg/ml) in six replicates and shown through the relative standard deviation - RSD (%). The detection limit (LOD) was defined as the amount of phenolic compounds which produces the signal three times the noise signal. The quantification limit (LOQ) is the amount of phenolic compounds produces a signal ten times the noise signal. The LODs were determined by adding 100 ml of phenol compounds mixture standard to the concentration of 1.0 mg/ml, in 0.5 g of the sample in six replicates and the LOQs was calculated.

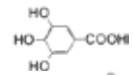
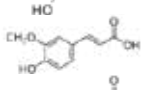
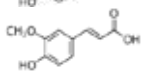
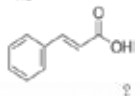
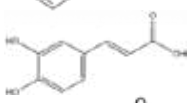
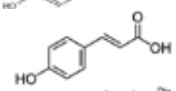
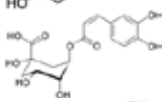
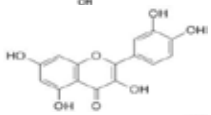
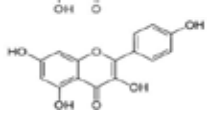
RESULTS

The method was evaluated in terms of linearity and repeatability, LOD and LOQ for ferulic, trans-cinnamic, 2-hydroxy cinamic, gallic, caffeic, p-coumaric and chlorogenic acid, quercetin and kaempferol.

HPLC-DAD chromatogram of standard solutions of the phenolic compounds preparing in mobile phase was shown in Figure 1.

Some of the validation parameters are shown in Table 1. The obtained LODs for all investigated phenolic compounds were 0.01 μg/ml with the LOQs of 0.03 μg/ml.

Table 1. R^2, repeatability (RSD, %) and structural formula investigated phenolic compounds

Phenolic acid	Structural formula	Retention time (min)	R^2	Repeatability (RSD, %)
Gallic acid		4.58	0.9998	6.76
Ferulic acid		22.48	0.9966	2.42
2 hydroxy cinnamic acid		24.00	0.9967	9.61
Trans cinnamic acid		26.43	0.9949	5.09
Caffeic acid		13.29	0.9984	9.48
p-Coumaric acid		18.94	0.9985	2.74
Chlorogenic acid		11.21	0.9996	4.18
Quercetin		40.75	0.9992	7.25
Kaempferol		37.67	0.9996	11.93

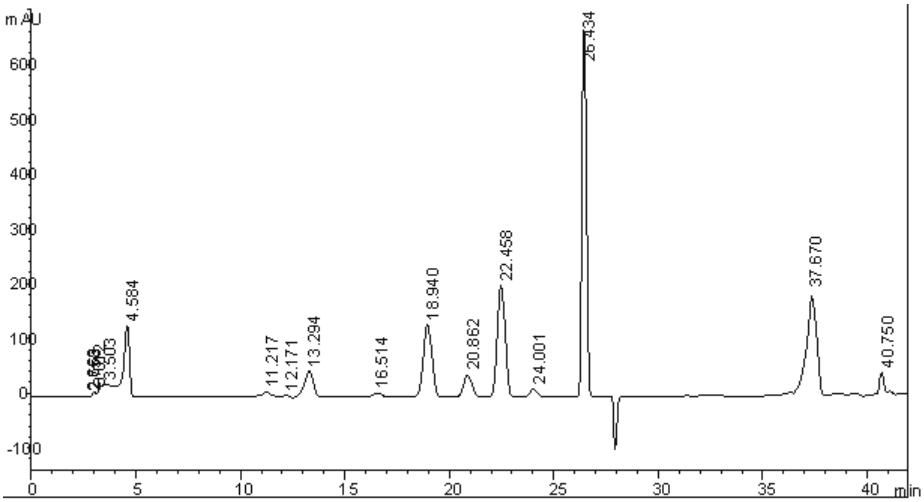


Figure 1. HPLC-DAD chromatogram of phenol compounds

Some of the calibration curves (gallic acid, caffeic acid, 2-hidroxycinnamic acid and kaempferol) were shown in Figure 2.

DISCUSSION

Phenolic compounds are found throughout the plant kingdom but the type of compounds present varies significantly depending on the taxonomic affiliation (Lattanzio, 2006). Extensive studies have been carried out to identify plant phenols with insecticidal properties and in *in vitro* experiments of Wójcicka (2010), phenolic compounds exerted negative influence on the feeding, reproduction, growth and survival of the *Sitobion avene, Schizaphis gramini* and *Mysus persicae*. Based on the large number of literature data, the most commonly found phenolic acids, with proved insecticidal and/or antifeedant activity, in plant extracts are quercetin and kaempferol, gallic, feruic and caffeic acids (Patton et al., 2005; Anonymous 1, 2006; Mesbah et al., 2007; Pavela, 2011; Zhang et al., 2013; Ladhari et al., 2013). Quercetin is one of the most abundant flavonoids and the defense secondary metabolite in plants. Liu-Shou-Zhu et al. (2007) suggest that quercetin at low concentrations can cause immune response of *Tenebrio molitor*. According to Mesbah et al. (2007), quercetin extracted from sunflower plants caused abnormal behavior, namely feeding arrest, growth inhibition and development retardation of *Spodoptera littoralis* larvae, deformation of pupae, moths and reduction up to 50% of laid eggs. Ismail et al. (2005) also described the antifeeding effects of well known flavonol glycosides, such as quercetin-3-O-glucopyranosides and kaempferol, while Mitchell et al. (1993) presented results indicating that among others, kaempferol and quercetin inhibit ecdysone 20-monooxygenase activity of *Aedes aegypti* adult females, wandering stage larvae of *Drosophila melanogaster* and pre-wandering and wandering stage of *Manduca sexta* larvae. All this implicated that mentioned compounds may function as biopesticides affecting insect ecdysteroidogenesis. It has become a common practice in USA to use C8-C12 alkyl ester of gallic acid to protect wooden material from insects feeding (Anonymous, 2006). However, according to Patton et al. (2005) caffeic acid expressed antifeeding activity towards adult Japanese beetles (*Popillia japonica*). Singh at al. (2014) represent that tomatoes are rich source of secondary metabolites, and its hairy roots efficiently produce phenolic compounds, such as rutin,

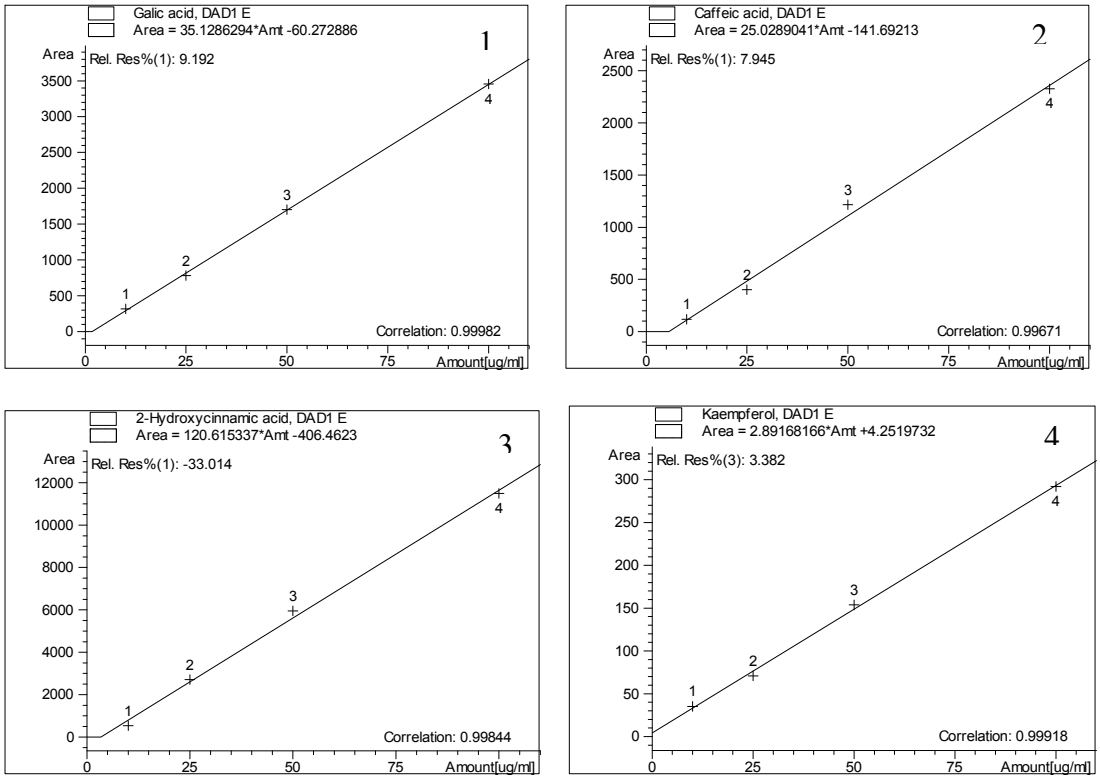


Figure 2. Calibration curves (1-gallic acid, 2-caffeic acid, 3-2-hidroxycinnamic acid, 4- kaempferol)

quercetin, kaempferol, gallic acid, protocatechuic acid, ferulic acid, chlorogenic acid, and caffeic acid. At 100 μL/g concentration, the phenolic compounds caused 53.34 and 40.00% mortality of *Helicoverpa armigera* and *Spodoptera litura*, respectively, after 6 days, while surviving larvae of both species, after 6 days showed 85.43 and 86.90% growth retardation, respectively. Also, chlorgenic acid, detected in extracts used in this work, has been described as an antifeedant and digestibility reducer in aphids (Miles and Oertli, 1993).

- In order to estimate the biochemical basis of plant extracts an efficient, sensitive and reliable HPLC-DAD method was validated. It can be applied to ferulic, trans-cinnamic, 2-hydroxy cinamic, gallic, caffeic, p-coumaric and chlorogenic acid and quercetin and kaempferol in the analysis of real plant extract samples.
- The obtained LODs for all investigated phenolic compounds were 0.01 μg/ml with the LOQs of 0.03 μg/ml.
- The correlation coefficient of all phenolic compounds were R^2>0.99 in the concentration range from 10.0 to 100.0 μg/ml with the repeatability RSD less than 11.93%.

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