Influence of fertilization in total polyphenol content in aniseed post-distillation waste material

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Anise (*Pimpinella anisum* L.), is a herbaceous plant from Apiaceae family. Its fruit is used in the pharmaceutical industry and in everyday nutrition because of its numerous benefits. It contains essential oil with *trans*-anethole as the main compound, which gives it that characteristic aroma. However, essential oil content in aniseed ranges around 3-4%, and the large amount of post-distillation waste material remains unused, but it can be a potential source of biologically active compounds. The aim of the present study was to investigate the total polyphenol content and antioxidative activity of anise post-distillation waste material, as well as the influence of fertilization on it. For this investigation the anise was grown during 2013, with the application of six different fertilization regimes (control, Slavol, BactoFil B-10, Royal Ofert biohumus, vermicompost and NPK). The content of total polyphenols remains after Clevenger oil distillation was determined by using Folin-Ciocalteau reagent, and expressed in mg/g gallic acid equivalents to dry extract (GAE). Further analysis of anise extracts was performed by LC-DAD-MS for identification phenolic compounds. Capacity of neutralization of free radicals samples was determined by DPPH method, and results are expressed in mg/mg Trolox equivalent to dry extract (TE). The results indicated that fertilization significantly increased content polyphenols total in anise post-distillation waste material, from 20.0 to 26.4 mg/g, in comparison to the control (11.6 mg/g). Hydroxybenzoic and hydroxycinnamic acids and their derivatives were identified in anise post-distillation waste extract, as well as flavanone and flavonol glycosides and trihydroxy flavonol derivates. However, antioxidant activity of all anise extract according to DPPH method is poor, and EC_{50} value ranges between 0.04 and 0.05 TE.

Keywords: *Pimpinella anisum*; oil cake; DPPH; fertilization; polyphenol; antioxidative activity.

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1. Introduction:

Anise (*Pimpinella anisum* L.) is a herbaceous plant from Apiaceae family. Its fruit (*Anisi fructus*) is used in the pharmaceutical industry and in everyday nutrition because of its numerous benefits (Acimovic and Dojcinovic 2014; Acimovic et al. 2015). It contains essential oil with *trans*-anethole as the main compound, which gives it that characteristic aroma (Orav et al. 2008).

Many aspects of the fertilization of anise have been extensively studied, focusing much attention on the seed yield (Gomaa and Abou-Aly 2001; Nabizadeh et al. 2012; Aćimović 2013), as well as all aspects related to their quality features, especially essential oil content (Carrubba and Ascolillo 2009). According to the results obtained in Turkey, fertilization influenced essential oil content, but the differences are not statistically significant (Faravani et al. 2013; Doğramaci and Arabaci 2015). Another research conducted in Egypt indicated that in arid conditions application of phosphorus fertilizer significantly increases essential oil content in a dose dependent level (Khalid 2012). Our previous research indicated that weather conditions during the year significantly influence essential oil content in aniseed, while different sources of fertilizers did not have a statistically significant effect on the essential oil content and its composition (Aćimović et al. 2015).

However, essential oil content in aniseed ranges around 3-4%, and the large amount of post-distillation waste material remains unused; however, it can be a potential source of biologically active compounds (Gavaric et al. 2015). Currently, there is great interest in the study of antioxidant substances mainly due to the findings concerning the effects of free radicals in the organism. The phenolic plant compounds have attracted considerable attention for being the main sources of antioxidant activity in spite of not being the only ones. The antioxidant activity of phenolics is a result of their redox properties (Aiyegoro and Okoh 2009).

The search for new antioxidants from waste materials has become the centre of attention in the last couple of years. The aim of the present study was to investigate the total polyphenol content and antioxidative activity of anise post-distillation waste material, as well as the influence of fertilization on it.

2. Materials and methods:

2.1. Growing practices

For this investigation the anise was grown during 2013, on experimental fields in Mošorin (45°18′05″N; 20°09′32″E). The influence of six treatments: Slavol, Bactofil B-10, Royal Ofert, vermicompost, NPK (15:15:15), and control (without fertilization) was examined. Slavol and Bactofil B-10 are microbiological fertilisers. Slavol (Agrounik) contains *Azotobacter chroococcum, A. vinelandi, Derxia sp. Bacillus megaterium, B. lichenformis, B. subtilis*. Bactofil B-10 (BioFil KFT) contains *Azotobacter vınelandı, Azospırıllum brasılense, A. lıpoferum, Bacıllus megaterıum, B. suptılıs, B. cırkulans, B. polymıxa, Pseudomonas fluorescens*. Apart from bacteria, these fertilisers contain natural vitamins and growth stimulator. However, the main difference between these two

fertilizers is time of application and mechanism of action. Slavol (in dose of 7 l/ha) is applied by watering during vegetation, time after time (twice), while Bactofil B-10 (in doses of 1.5 l/ha) is applied through soil before sowing. Royal Ofert biohumus (Altamed) is specific organic manure, made from organic waste from poultry and pig farms inoculated with domestic fly larvae, while vermicompost (PR Ivić) is modified cattle manure with *Lumbricus terrestris*. Both of this fertilizers are applied before sowing of anise seeds in dose of 3 t/ha and 5 t/ha, respectively. These four fertilizers are allowed for applications in organic cropping system. However, the chemical fertilizer NPK is often used in conventional agriculture. The dose of applications this fertilizer was 400 kg/ha in formulation 15:15:15.

The crop is established in April, by direct sowing with row spacing of 0.35 m, respecting density of 200 plants/m^2. Weeds were controlled by hoeing and weeding when needed. Measures of protection from diseases and insects were not used. Harvest was carried out by hand in phase of full maturity. Data for meteorological conditions during growing season 2013 is shown in Figure 1.

2.2. Essential oil isolation

The dried ripe seeds were subjected to hydro-distillation using an all glass Clevenger-type apparatus to extract essential oils according to the method outlined by the European Pharmacopeia (2004). The samples were ground, homogenized and made into a fine powder. In order to extract the essential oils, 100 g of the powder was placed in 1 l conical flask and connected to the Clevenger apparatus. 500 ml of distilled water was added to the flask and heated to the boiling point. The steam in combination with the essential oils was distilled into a graduated cylinder for 4 h and then separated from aqueous layers.

2.3. Determination of the total polyphenols

The number of total phenolics in water soluble extracts from post-distillation waste material was determined to use the Folin-Ciocalteu reagent according to the method with gallic acid as a standard (Slinkard and Singleton 1977). The results were expressed as milligrams of gallic acid equivalent per 1 gram of fresh weight, (mg GAE/g).

2.4. Characterisation of phenolics compounds

The water soluble extracts of aniseed which remains after the essential oil distillation were dissolved in methanol to an approximate concentration of 5 mg/ml. The LC/DAD/MS analyses were carried out by an Agilent 1200 HPLC instrument (Agilent Technologies, Waldbronn, Germany) with a binary pump, an autosampler, a column compartment equipped with a Zorbax Eclipse Plus C18 column (1.8 µm, 4.6 mm × 150 mm, Agilent Technologies) and a diode-array detector coupled with a 6210 time-of-flight LC/MS system (Agilent Technologies). The mobile phase consisted of water containing 0.2% formic acid (A) and acetonitrile (B). The combination of isocratic and gradient modes of elution was used as follows: 0–1.5 min 5% B, 1.5–26 min, 5–95% B, 26–35 min, 95% B. The mobile phase flow rate was 1.4 mL/min, the column temperature was 40 °C and the injection volume was 5 µl. Spectral

data from all the peaks were accumulated in the range of 190–450 nm and chromatograms were recorded at 260, 280, 290 and 320 nm. MS-data were collected by applying the following parameters: ionization negative ESI, capillary voltage 4000 V, gas temperature 350 °C, drying gas 12 L/min, nebulizer pressure 45 psi, fragmentary voltage 140 V, mass range 100–2000 m/z. A personal computer system running MassHunter Workstation software was used for data acquisition and processing. Phenolic compounds were detected as [M−H]− or [2M−H]− signals using these parameters. Compounds were characterized by their retention times (*tr*), mass spectra and UV spectra, and were tentatively identified based on the previous data published by other authors. Their complete identification was not possible since the full scan mass spectra of the chromatographically separated compounds gave only deprotonated [M−H]− ions, and MS/MS experiments were not possible with the instrumentation used.

2.5. Antioxidant activity

The antioxidant activity of the samples was evaluated by means of the 2,2-diphenyl-1-picrylhydrazil (DPPH) radical scavenging method. This spectrophotometric assay uses stable DPPH radical as a reagent. The methanolic solution of the investigated sample (200 µl) (with starting concentrations of 200, 300, 400, 500 µl/ml of solution) was added to the 1800 µl methanolic solution of DPPH radical (concentration of 0.04 mg/ml) and after shaking, the reaction mixture was left to react in the dark for 30 min at room temperature. Then, absorbance of the remaining DPPH radical was measured at 517 nm on Cintra 40 UV–Visible spectrophotometer. Every concentration was done in triplicate and the same experiment was done with Trolox standard, a well-known synthetic antioxidant. The results were expressed as mM of trolox equivalent per 1 g of fresh weight, (mM TE/g).

The concentrations of the extracts which reduce the absorption of DPPH solution by 50% (EC_{50}) were obtained from the curve dependence of the absorption of DPPH solution at 517 nm from the concentration for each extract and a standard antioxidant. Origin 8.0 software was used to calculate these values. Tests were carried out in triplicate.

2.6. Statistical analysis

Data was subject to statistical analysis using the program package Statistica 10 (StatSoft Inc., 2011, University of Novi Sad license) and was expressed as mean value. The differences in main values were compared using analysis of variance (ANOVA) followed by the least significant difference (LSD) test at $P<0.05$. Every experiment was performed in triplicate.

3. Results and discussion

3.1. Results

The results indicated that fertilization significantly increased the total polyphenol content in anise post-distillation waste material, from 20.0 to 26.4 mg/g, in comparison to the control (11.6 mg/g) (Figure 2). As it can be seen, out of all of the applied fertilizers the least efficient was the

vermicompost, then BactoFil B-10 (22.3 mg/g), Royal Ofert (23.1 mg/g) and Slavol (23.4 mg/g), while the most efficient was the application of the NPK fertilizer.

Phenolic compound such as hydroxybenzoic and hydroxycinnamic acids and their derivates were identified in anise post-distillation waste extract, as well as flavanone and flavonol glycosides and trihydroxy flavonole derivates (Table 1). However, antioxidant activity of all anise extracts according to DPPH method is poor, and EC_{50} value ranges between 0.04 and 0.05 TE (Figure 3).

3.2. Discussion

Different sources of fertilizers, varieties and their interaction were found to have a significant effect on phytochemical content. The phenolic and flavonoid content were significantly higher in the tubers of cassava (*Manihot esculenta* Crantz) with the vermicompost treatment compared to mineral fertilizer (Omar et al. 2012). However, the results of the study with bush tea (*Athrixia phylicoides* L.) demonstrated that the application of nitrogenous, phosphorus and potassium fertilizers increased the total polyphenol content in leaves regardless of the season (Mudau et al. 2007). Furthermore, the application of microbiological fertilizers based on *Bacillus* sp. influences the increase of total polyphenol content in grapes (Sivčev et al. 2005). Investigations with another plant from Apiaceae family, dill, show a significant increase in total flavonoids content with the application of nitrogen fertilizer and bio-fertilizer, while the maximum increase was noticed with bio-fertilizers combined with nitrogen fertilizer (Aly et al. 2015).

From the other side, the polyphenol constituents of anise fruit are: quercetin 3-glucuronide, rutin, luteolin 7-glucoside, isoorientin, isovitexin, apigenin 7-glucoside and a luteolin glycoside (Kunzemann and Herrmann 1977).

Investigations show that the antioxidant properties of water extract of anise seed exhibited greater antioxidant capacity than that of ethanol (Gülçın et al. 2003). However, ethanolic extracts of aniseed exhibited DPPH radical scavenging activity in a concentration dependent manner (Rajeshwari et al. 2011). From the other side, extracts of waste water after hydro distillation of *Satureja cuneifolia* and *S. montana*, as well as *Thymus vulgaris* showed good ability to reduce stable DPPH radical (Ćavar et al. 2013, Gavarić et al. 2015).

4. Conclusions:

The essential oil content in aniseed usually varied between 3 and 4%. However, a large amount of post-distillation waste material which remains unused contains between 11.6 and 26.4 mg GAE/g of total polyphenols, and possesses a poor antioxidative capacity (0.04-0.05 TE/g). Fertilization as agro-technical practice significantly increases the total polyphenol content whereas no significant effect was shown on antioxidant activity. Further research will be focused on agro-food implementation of the post-distillation waste material of aniseeds and other plants which are used for the essential oil production.

Table 1. The phenolics compounds of aniseeds post-distillation waste identified by LC/DAD/MS

No	t_r (min)	MW	MF	λ (nm)	ID compound (based on literature data)	Literature source
1	1.05	504,1760	$C_{24}H_{24}O_{12}$	212; 242;304	Luteolin-3'-(3"-O-acetyl)-O-glucuronide; or Luteolin-3'-(4"-O-acetyl)-O-glucuronide	Perez-Fons et al. 2010
2	1.06	320,0426	$C_{12}H_{18}O_3$		Jasmonic acid	Babovic et al. 2010
3	1.06	504,1760	$C_{24}H_{24}O_{12}$		Luteolin-3'-(3"-O-acetyl)-O-glucuronide; or Luteolin-3'-(4"-O-acetyl)-O-glucuronide	Perez-Fons et al. 2010
4	1.08	210,0426	$C_{12}H_{18}O_3$	218; 278; 338	UNC	
5	1.22	276,0317	$C_6H_{12}O_{12}$		UNC	
6	1.25	134,0225	$C_4H_6O_5$		UNC	
7	1.29	104,0116	$C_3H_4O_4$	200; 216;282	UNC	
8	1.43	192,0285	$C_6H_8O_7$	216; 260	UNC	
9	2.59	300,0858	$C_{13}H_{16}O_8$	212sh; 248	Hydroxybenzoic acid 4-O-glucoside	Parejo et al. 2004
10	3.61	316,0806	$C_{13}H_{16}O_9$	200; 206sh; 218; 254; 292sh	Protocatechuic acid 4-O-glucoside	Vallverdu-Quetalz et al. 2014a
11	3.67	432,1288	$C_{21}H_{19}O_{10}$	218sh; 250; 296	Apigenin 7-O-glucoside	Hossain et al. 2010
12	4.28	488,1552	$C_{21}H_{28}O_{13}$	202sh; 212; 218; 258	UNC	
13	4.44	354,0972	$C_{16}H_{18}O_9$	242; 296sh; 326	Horogenic acid	Vallverdu-Quetalz et al. 2014b
14	4.58	358,1285	$C_{16}H_{22}O_9$		UNC	
15	4.58	348,0999	$C_{21}H_{32}O_4$		Salviviridinol	Babovic et al. 2010
16	4.88	292,0712	$C_{18}H_{12}O_4$		UNC	
17	5.13	300,0862	$C_{13}H_{16}O_9$		Hydroxybenzoic acid 4-O-glucoside	Parejo et al. 2004
18	5.15	252,0285	$C_{11}H_8O_7$		UNC	
19	5.28	138,0275	$C_7H_6O_3$	212; 256	Hydroxybenzoic acid	Vallverdu-Quetalz et al. 2014b
20	5.37	176,0691	$C_7H_{12}O_5$	228; 296; 332	UNC	
21	5.37	354,0965	$C_{16}H_{18}O_9$	228; 296; 332	Horogenic acid	Vallverdu-Quetalz et al. 2014b
22	5.49	326,1024	$C_{15}H_{18}O_8$		O-hexozide-Coumaric acid	Vallverdu-Quetalz et al. 2014b
23	5.54	354,0970	$C_{16}H_{18}O_9$	202sh; 218; 284; 330	Horogenic acid	Vallverdu-Quetalz et al. 2014b
24	5.61	426,1762	$C_{17}H_{30}O_{12}$		UNC	
25	5.69	368,1130	$C_{17}H_{20}O_9$		Feruoilhinonic acid	Kaiser et al. 2013
26	5.93	180,0430	$C_9H_8O_4$	216; 242; 294sh; 326	Coffeic acid	Vallverdu-Quetalz et al. 2014b
27	5.93	294,0368	$C_{18}H_{30}O_3$	216; 242;	9-okso-10(E),12(Z)-	Babovic et al. 2010

				294sh; 326	oktadecadienonic acid	
28	5.95	316,1167	$C_{16}H_{12}O_7$		Isorhamnetin	Hossain et al. 2010
29	6.04	402,1546	$C_{15}H_{26}O_{10}$		UNC	
30	6.05	198,0891	$C_9H_{10}O_5$		Siriginic acid	Vallverdu-Quetalz et al. 2014b
31	6.11	154,0270	$C_7H_6O_4$		Procatehinic acid	Vallverdu-Quetalz et al. 2014b
32	6.30	198,0901	$C_9H_{10}O_5$	228; 292	Siriginic acid	Vallverdu-Quetalz et al. 2014b
33	6.49	580,1448	$C_{26}H_{28}O_{15}$	200; 212; 258sh; 270; 348	Quercetin-O-xylo-pentozide	Singh et al. 2011
34	6.70	448,1027	$C_{21}H_{20}O_{11}$	254; 268sh; 346	Kamferol-3-O-glukozide	Vallverdu-Quetalz et al. 2014b
35	6.74	368,1125	$C_{17}H_{20}O_9$	214; 272; 348	Feruoilhinonic acid	Kaiser et al. 2013
36	6.74	267,0760	$C_{13}H_9N_5O_2$ $C_{12}H_{13}NO_6$	214; 272; 348	UNC	
37	6.98	416,1708	$C_{19}H_{28}O_{10}$	214; 266; 298sh	UNC	
38	6.98	594,1608	$C_{27}H_{30}O_{15}$	214; 266; 298sh	Apigenin-C-dyhexozide	Vallverdu-Quetalz et al. 2014b
39	7.08	208,0383	$C_{10}H_8O_5$	222; 284; 290sh	UNC	
40	7.09	276,0761	$C_{18}H_{12}O_3$	222; 284; 290sh	UNC	
41	7.14	564,1499	$C_{26}H_{28}O_{14}$	214 sh; 228; 274sh; 314	Apigenin-7-apiozilglucozide	Justensen 2000
42	7.32	504,1865	$C_{24}H_{24}O_{12}$	274sh; 260; 342	Luteolin-3'-(3"-O-acetyl)-O-glucuronide; or Luteolin-3'-(4"-O-acetyl)-O-glucuronide	Perez-Fons et al. 2010
43	7.44	432,1076	$C_{21}H_{20}O_{10}$	216; 272; 342	Apigenin 7-O-glucoside	Vallverdu-Quetalz et al. 2014b
44	7.65	472,1593	$C_{21}H_{28}O_{12}$	226sh; 258	UNC	
45	7.95	516,1283	$C_{25}H_{24}O_{12}$	250; 296sh; 330	Dicafenoilhinonic acid	Parejo et al. 2004
46	7.95	566,1653	$C_{19}H_{34}O_{19}$	250; 296sh; 330	UNC	
47	8.22	408,1446	$C_{20}H_{24}O_9$ $C_{13}H_{28}O_{14}$	242; 290sh; 332	UNC	
48	8.22	516,1296	$C_{25}H_{24}O_{12}$	242; 290sh; 332	Dicafenoilhinonic acid	Parejo et al. 2004
49	8.98	362,1148	$C_{22}H_{18}O_5$		UNC	
50	8.98	372,1435	$C_{17}H_{24}O_9$		UNC	
51	9.28	138,0312	$C_7H_6O_3$	204; 238; 302	p-Hydroxybenzoic acid	Vallverdu-Quetalz et al. 2014b
52	9.60	200,1056	$C_{10}H_{16}O_4$		UNC	

tr – Retention time, MW – Molecular Weight, MF – molecular formula, λ – Absorbance maxima, UNC – Unknown compound

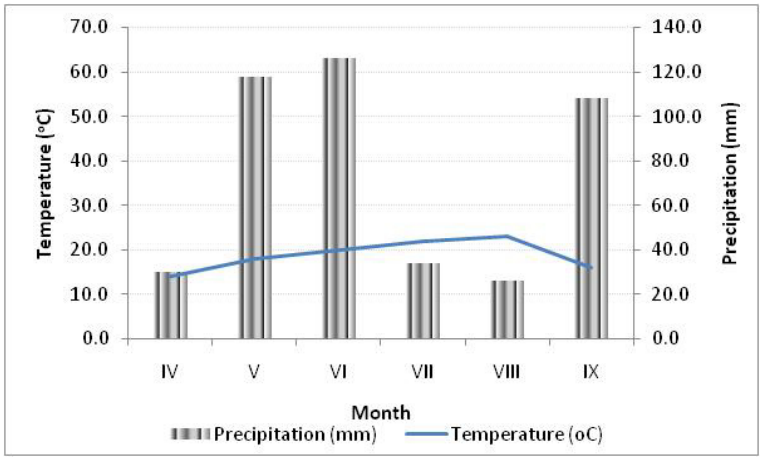

Figure 1. Weather conditions during anise growing season 2013

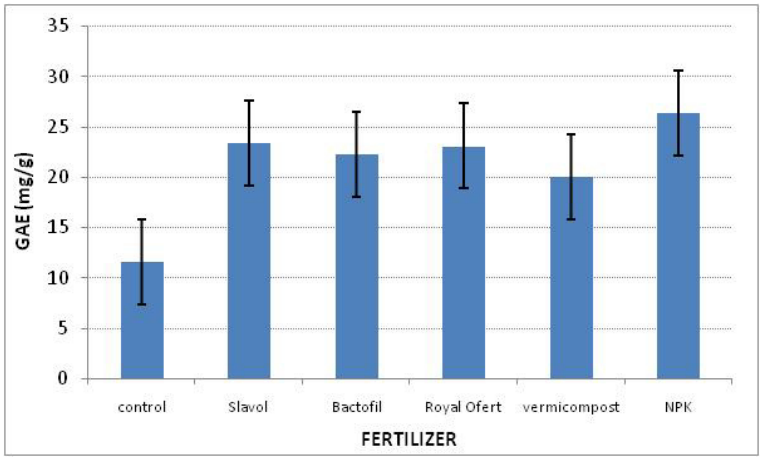

Figure 2. The total polyphenol content in anise post-distillation waste material (mg GAE/g dry extract) depend on apply fertilizer

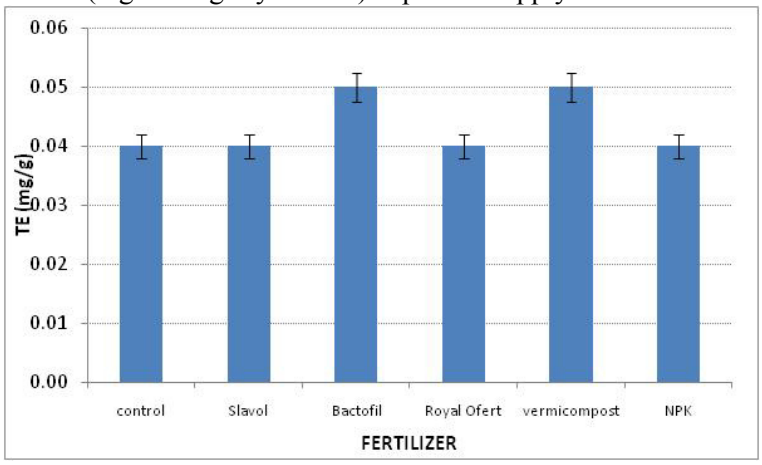

Figure 3. The antioxidant activity of anise post-distillation waste material (mM TE/g) depend on apply fertilizer

Competing interests

The authors declare that they have no competing interests.

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