OPEN ACCESS Potravinarstvo® Scientific Journal for Food Industry

Potravinarstvo, vol. 9, 2015, no. 1, p. 382-387
doi:10.5219/503
Received: 27 July 2015. Accepted: 15 October 2015.
Available online: 30 October 2015 at www.potravinarstvo.com

ISSN 1337-0960 (online)

STUDY OF ANTIOXIDANT AND ANTIMICROBIAL PROPERTIES OF GRAPEVINE SEEDS, GRAPE AND ROSEHIP PRESSINGS

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ABSTRACT

In our experiment, we studied the antimicrobial and antioxidative effect of phytogenic additives. Three additives (grapevine seeds, grape and rosehip pressings) were selected to be monitored. The extracts about concentrations of 1:3 and 1:5 were prepared from them. The monitoring of antimicrobial properties was focused on the pathogenic bacteria *Clostridium perfringens* and *Escherichia coli* causing a serious disease in avian species. The bacteria were prepared in the dilutions of 10^2, 10^4 and 10^6. The antimicrobial effect was observed in the inhibition zones. The antioxidant activity was determined using DPPH method within the antioxidant analysis. Furthermore, the content of flavanols, hydroxycinnamic acids and the total content of polyphenolic compounds was also determined. In the monitoring of the antimicrobial effect of grapevine seeds, grape and rosehip pressings at *E. coli*, a reduced growth of KTJ (colony forming units) was observed in the disk area during the dilution of 10^6 and 10^4. Reduced growth of *C. perfringens* at a dilution of 10^6 was noticed using the extracts of grapevine seeds and grape pressings. Low reduced growth of *C. perfringens* at a dilution of 10^6 was found out using rosehip pressings. In a dilution of 10^2 and 10^4 in *C. perfringens* and 10^2 in *E. Coli,* a very low increase of KTJ was observed therefore the zones of inhibition were not possible to measure. In all monitored additives, the antimicrobial effect was proved. The additives reduced the growth of pathogenic *E. coli* and *C. perfringens.* Within the antioxidant analysis, the highest antioxidant activity was found out in grapevine seeds (7.021 $g.L^{-1}$ GAE), which also contained the highest content of flavanols (3000 times higher than the rosehip pressings and 300 times higher than grapevine seeds pressings), hydroxycinnamic acids (1000 times higher than in grape pressings and 7600 times higher than in rosehip pressings) and the total content of polyphenolic compounds (580 times higher than grape pressings and 2000 times higher than the rosehip pressings) of the monitored additives.

Keywords: *Clostridium perfringens; Escherichia coli*; grapevine seeds; pressings; rosehip

INTRODUCTION

Natural products are an important source of phenolic compounds. The interest in the compounds is in their ability to bond the important free radicals causing lipid oxidation, which is the main factor of lower quality of foods during the processing and storage. Furthermore, they have an important role in the progression of a wide variety of diseases such as cancer, atherosclerosis, inflammation and aging depending on the formation of free radicals. Catechin and resveratrol are the two most frequent phenols present in natural products. Catechin is found out in significant quantities, for example grapes, apples and tea. Phenolic compounds are extracted from grape extracts commonly used as active ingredients in the manufacture of pharmaceutical products. These compositions are used in skin preparations for the treatment of hemorrhoids, or to reduce platelet aggregation and oxidation abilities. Their effectiveness as preservatives was demonstrated in peeling fruit and vegetables, juices and other natural products **(Pinelo et al., 2005)**. Phenolic compounds may affect the growth and metabolism of bacteria. They cause the activating or inhibitory effect on the growth of microorganisms according to their composition and concentration **(Vaquero et al., 2007a)**. Some studies have shown that phenolic compounds, present in the wine, can influence bacterial growth and metabolism but the antimicrobial effect depends on the particular compound **(Ganan et al., 2009)**. The content of phenolic compounds in natural materials is quite variable. It depends on the particular crop species but also their varieties. Their content is conditioned genetically and influenced by climatic and agronomic conditions. Changes in the content of phenolic compounds largely also indicates germination, degree of ripeness as well as technical processing and storage of plant products **(Boncikova et al., 2012)**. After making wine, about 20 % of grapes remain in the form of skin pressings, seeds and stalks. The pressings contain significant quantities of phenolic compounds, which are not extracted into the wine. Resveratrol, present in wine in small quantities, was probably the most studied flavonoid. The phenolic compound inhibits the growth of microbial species (*S. aureus, Enterococcus faecalis, and Pseudomonas aeruginosa*) and several pathogenic fungi as dermatophytes causing skin infections and also inhibits the biofilm formation of *E. coli* and *P. aeruginosa*, *V. cholerae*. It appears that resveratrol has antimicrobial, antiparasitic and anti-inflammatory effects showing the potential to be used for microbial food safety against the infections endangering people **(Friedman, 2014)**. The applications of grape pressings in food technology

demonstrated the potential use of oil with an effective antioxidant capacity as an inhibitor of lipid oxidation in fish, frozen fish muscle and cooked, chilled turkey meat during storage **(Goni et al., 2007, Horky et al., 2013)**. The antioxidants, occurring in grapevines, are phenolic acids (benzoic acid and hydroxycinnamic), stilbene derivatives, flavan-3-ols (catechin and epicatechin), flavonols (quercetin and myricetin), and anthocyanidins. The antioxidant potential of grape seed is twenty times higher than E vitamin and fifty times higher than C vitamin which is obvious from higher levels of polyphenols, proanthocyanidins and units of flavan-3-ol oligomers particularly catechin and epicatechin occurring in the extract of grapevine seeds. However, the use of natural antioxidants in the diet of animals could be limited by the low bioavailability of polyphenols **(Brenes et al., 2010; Horky, 2014; Horky et al., 2012)**. Another interesting source may be rosehip fruits **(Loetscher et al., 2013)**. Currently, rosehip is widely used as aromatic and medicinal plant with high antioxidant activity **(Yesilbag et al., 2011)** In vitro tests showed high antioxidant capacity of the rosehip especially in the lipophilic fraction extract, which is probably caused by the content of phenolic compounds **(Loetscher et al., 2013)**.

Rosehip has long been used in many European countries such as herbal teas, vitamin supplements or food products as it contains large amounts of vitamin C. Except to ascorbic acid, rosehips contain also carotenoids and phenolics, which are also the important antioxidants **(Gao et al., 2000)**.

MATERIAL AND METHODOLOGY

Experimental design

Three phytogenic additives were selected to be monitored - grapevine seed (Marlen variety) grape and rosehip pressings. The effect of selected additives was determined using laboratory methods. Their antimicrobial and antioxidant effects were also found out.

Microbiological analysis

Extract preparation

The extracts were prepared from grape seeds, grape and rosehip pressings. The samples of the plant additives were first dried at 45 °C to a constant weight and then milled. The extracts were prepared at two concentrations of 1:3 (30 g sample +90 mL water) and 1:5 (20 g +100 mL water). Weighed samples were filled with boiled water and then placed in a water bath at 95 °C for 1 hour. After removal from the bath, the samples were centrifuged on a centrifuge (Biosan, Latvia) at 1500 rpm for 20 minutes.

Microbe preparation

For *Escherichia coli* bacterium, a pure culture of the Czech Collection of Microorganisms was used. *Clostridium perfringens* was grown on a selective medium - Tryptone neomycin sulfite agar (TSN) for the microorganism at 46 °C for 24 hours. For each bacteria, the suspension was prepared in the application of a microbe in a saline solution with a density of 1 McFarland (108). Subsequently, three dilutions (10^2, 10^4, 10^6) were prepared in decimal dilutions. Petri dishes with Violet Red Bile agar (VRBL) - *E. coli* and sulfite neomycin Tryptone agar (TSN) - *C. perfringens* was inoculated with 0.1 mL of suspension with sterile pipette and spread using sterile stick. Violet red bile agar (VRBL) contains in 1 liter of medium: 7.0 g peptic digest of meat, 3.0 g yeast extract, 10 g lactose, 1.5 g bile salts, 5.0 g sodium chloride, 30 mg neutral red, 2 mg crystal violet, 12.0 g bacteriological agar. Tryptone sulfite neomycin agar (TSN) contains in 1 liter of medium: 15 g, 10 g tryptone yeast extract, 1 g sodium sulfite, 0.5 g ferric ammonium citrate, 50 mg neomycin sulfate, 20 mg polymixin B sulfate, 13.5 g bacteriological agar. The extracts were pipetted in quantities of 30 µL onto sterile paper disks of 9 mm in diameter, which were then placed on a petri dish. Petri dish with *C. perfringens* was then inserted into the anaerostat (Merck, Germany) with the generator of anaerobic environment - Anaerocult A (Merck, Germany) and placed in a thermostat of 46 °C for 24 hours. Petri dishes with *E. coli* were put into a thermostat at 37 °C for 24 hours. Two repetitions were prepared in each dilution. After the incubation, the inhibition zones were measured using ruler (weakened growth zones of KTJ - colony forming units of bacteria) around the disks.

Antioxidant parameters

Determination of antioxidant activity by DPPH

The radical solution of DPPH (2,2-diphenyl-β-picrylhydrazyl radical) of 2000 µL was dosed in 3 mL cuvette using pipette and m = 9.35 mg of DPPH radical was weighed. The measured amount was put into a volumetric flask of 250 mL and supplemented with methanol. Then 40 ml of the sample was added. In this case, it was the sample of wine and left at 22 °C for 25 minutes. After a given time, the absorbance was measured at 505 nm. The calculation of the antioxidant activity was performed from the calibration curve, as a standard was used gallic acid (10 – 200 $mg.L^{-1}$). The results were expressed in $mg.L^{-1}$ of the antioxidant equivalents of gallic acid.

Determination of total flavanols

The preparation of reagents - 40 µL volume of sample was dispensed into 3 mL cuvette and then diluted in 1960 µL of reagent [(0.1 % DMCA = p-dymethylaminocinnamaldehyd) and 300 mL of MHCl in MeOH (methanol)]. The mixture was shaken and incubated for 12 minutes at the room temperature (about 22 °C). After 12 minutes, the absorbance was measured at the machine, called double beam spectrophotometer of SPECORD 210 brand, Carl-Zeis Jena, Germany, at λ = 640 nm against the empty cuvette. The results were expressed as catechin equivalents.

Determination of total hydroxycinnamic acids

The measurement was performed using SO_2 method. In a 2 ml vial, 200 µL sample with 1.8 mL and 1.1 mol HCl were shaken. Blank test to each sample was prepared in the same way. HCl solution was replaced with fresh 0.22 mol solution of $K_2S_2O_5$ (SO_2). After 180 minutes, the

absorbance of samples with HCl was measured in the cuvette at 280 nm. The results were calculated for values at $mg.L^{-1}$.

The calculation of the total hydroxycinnamic acids: OD280 = 10 * dilution * A (HCl) 280

Determination of total polyphenolic compounds

Wine sample of volume (50 mL) was pipetted into a cuvette and diluted with 1.5 mL ACS water. Subsequently, 0.05 mL of Folin-Ciocalt reagent (Sigma-Aldrich, US) was added. After 30 minutes at 22 °C, the absorbance was measured at dual beam spectrophotometer called SPEKOL 2000 at wavelength λ = 640 nm and λ = 670 nm against a blank sample (gallic acid). The results were expressed as gallic acid equivalents in mg/100 g.

RESULTS AND DISCUSSION

The experiment was aimed to find out the antioxidant and antimicrobial activity of grapevine seeds, grape and rosehip pressings. The task was also to determine the relationship between the antioxidant and antimicrobial activity. As markers of antioxidant potential were selected the contents of flavones, hydroxycinnamic acids, polyphenol compounds, DPPH. The main observed parametres from a microbiological point of view were *C. perfrigens* and *E. coli*, which are directly tied to the grapevine seeds, grape and rosehip pressings.

Determination of antimicrobial activity

The basic characteristics of plant additives is their wide antimicrobial activity **(Jakubcova et al., 2014a)**. Many studies proved a potential of use of various phytogenic additives into feed rations of poultry as alternative to antibiotics **(Jakubcova et al., 2014b)**.

The attention was focused on the study of two important pathogens such as *C. perfringens* and *E. coli* in the digestive tract of chickens. These microorganisms are responsible for serious poultry diseases. The antimicrobial activity was determined in the inhibition zones. Reduced growth of *C. perfringens* was found out very low using the extract of rosehip pressings at a dilution of 10^6. Reduced growth of *C. perfringens* was recorded using the extract of grapevine seeds and grape pressings at a dilution of 10^6. During the monitoring of the antimicrobial effect of the extracts of grapevine seeds and grape pressings for *E. coli*, a reduced growth of KTJ (colony forming units) was detected in the disc area at a dilution of 10^6 and 10^4. In the extracts of rosehip, a strong reduced growth of KTJ was recorded around the disc areas at a dilution of 10^4 and 10^6. For the dilutions of 10^2 and 10^4 in *C. perfringens* and 10^2 in *E. coli*, a low increase was observed in KTJ. The antimicrobial effect of the extracts was not possible to be evaluated. During the dilution of 10^4 in *C. perfringens* and 10^2 in *C. perfringens* and *E. coli*, a very low increase was found out in KTJ. The inhibition zones around the discs were impossible to be measured.

Vaquero et al., (2007b) demonstrated the antimicrobial effects of various concentrations of phenolic compounds contrary to *Listeria monocytogenes* in three types of wine. In this study, all samples of wine proved the inhibition zone contrary to bacteria, which was higher with increasing concentration of polyphenols. Further, it was demonstrated the antibacterial effect of three types of wine to the bacterium such as *Escherichia coli, Preteus mirabilis,* Serratia marcescens*, Flavobacterium sp. and Klebsiella pneumoniae, Staphylococcus aureus, Pseudomonas aeruginosa* **(Vaquero et al., 2007a)**. The extract of grapevine seed pressings showed a high antibacterial activity *in vitro* contrary to bacteria such as *S. aureus* and *E. coli*, and a low activity contrary to *Salmonella sp* **(Rotava et al., 2009)**. In our monitoring, we managed to prove that the pressings have the antibacterial effect on bacteria such as *E. coli* and *C.perfringens.* Based on the results of his study **(Viveros et al., 2010)** states that the products of grapes rich in polyphenols grapes affected the increase of beneficial bacteria in the ileum, as well as on increasing the villus height and crypt depth in the jejunum. These factors can have a significant impact on the physiology and biochemistry of cancer. The advances in the understanding of interactions between bioactive compounds in feed mixtures and specific intestine bacteria could contribute to a better understanding of positive and negative interactions *in vivo* and to identify new functional intestinal microorganisms. The study of **(Gadang et al., 2008)** demonstrated the antimicrobial effect in a combination of

Table 1 Averages of inhibition zones.

MO	Concentration MO [McF]	Dilution	Averages of inhibition zones [mm]					
			Grapevine seeds		Grape pressings		Rosehip pressings	
			1:3	1:5	1:3	1:5	1:3	1:5
C.perfrigens	1	10^6	14	12	9	10	9	11
		10^4	-	-	-	-	-	-
		10^2	-	-	-	-	-	-
E.coli	1	10^6	15	9	13	10	12	14
		10^4	16	11	12	16	11	12
		10^2	-	-	-	-	-	-

n=72
MO – microorganisms
McF – McFarland

nisin, malic acid and extract grapevine seeds to *L. monocytogenes*. The results of the study of **Vaz et al., (2012)** showed that wine proves a strong inactivation effect against vegetative cells of two strains of *B. cereus.* Wine also affects the viability of *C. jejuni*, while red and rose wines are more effective than white wines. However, alcohol reduces the survival rate of *C. jejuni*, phenolics also significantly affect their viability, especially p-hydroxybenzoic acid and gallic acid. Wine thus creates a hostile environment for the survival of this pathogen. It would be interesting to study the possible use of wine phenolic compounds as alternatives to the use of the antimicrobial growth promoters contrary to these bacteria in broilers **(Ganan et al., 2009)**.

Determination of antioxidant properties

Three major groups of substances - polyphenolic compounds, flavanols and hydroxycinnamic acids were selected to study the antioxidant properties. Further, the antioxidant activity represents the antioxidant power of antioxidant components.

Determination of antioxidant activity

DPPH method was used to determine the antioxidant activity. It is one of the most basic and most commonly used methods for the determination of the antioxidant activity. It is based on the reaction of a biological matrix (grape seeds and pressings in our case) with free radical DPPH. The results are then expressed in the equivalent amount of the antioxidant substances gallic acid – GAE in our experiment) which is capable of putting out the amount of the radical. In Table 2, the results of the antioxidant activity of observed samples are showed. The highest antioxidant activity was observed in the grapevine seeds. The measurement for the grapevine seeds was 500 times higher than for grape pressings and 575 times higher than for the rosehip pressings.

Determination of flavonols content

Flavonols are primarily synthesized in grape skin. In the red grapes, they are found out in smaller amounts than anthocyanins. Particularly, flavonols are important during co-pigmentation with anthocyanins **(Hilbert et al., 2015)**. For the flavanol determination, the method, based on a reagent reaction with DMAC, was used. The highest content of flavanols was measured with grapevine seeds, and almost 3000 times higher than the rosehip pressings and 300 times higher than grapevine seeds pressings. The content of flavanols in the monitored additives is shown in Table 3.

Determination of the hydroxycinnamic acid content

Hydroxycinnamic acids are the principal group of phenolic compounds located in the pulp of grapes. They occur in the form of esters of tartaric acid in the skin and pulp. The highest content of hydroxycinnamic acids was determined in the grapevine seeds. The measured values of grapevine seeds were 1000 times higher than in grape pressings and 7600 times higher than in rosehip pressings. The results of measurement of hydroxycinnamic acids content are given in Table 4.

Determination of the total polyphenolic compounds

Polyphenols create one of the most abundant and widely distributed group of natural products of the plant kingdom. They include a sufficient amount of molecules with polyphenolic structure but also molecules with one phenolic circle. Polyphenols contained in grapes and wine can generally be divided into two main groups: non flavonoids (hydroxybenzoic and hydroxycinnamic acids and their derivatives, and stilbene phenol alcohols) and flavonoids (anthocyanins, flavanols, flavonols and dihydroflavonol). Many of polyphenols have been identified in grape pressings, in which are the most abundant anthocyanins, flavanols, flavonols, hydroxybenzoic and hydroxycinnamic acids and stilbenes **(Antoniolli et al., 2015)**.

The highest content of total polyphenolic compounds has been measured in grapevine seeds. This value was almost 580 times higher than grape pressings and 2000 times higher than the rosehip pressings. For content of pressings coming from grapevine seeds, the value was measured 10 times higher than the rosehip pressings. The measured values are given in Table 5.

Spranger et al., (2008) evaluated the antioxidant activity of test compounds determining the trapping capacity of different types of radicals in his study. All test methods showed that polymeric procyanidins on an equimolar basis had the highest antioxidant activity followed by oligomeric procyanidins, while catechins reached a lower antioxidant activity than oligomers and polymers. The antioxidant activity of procyanidins of grape seeds positively related to their level of polymerization. Additionally, procyanidins showed greater antioxidant activity than other antioxidants as e.g. vitamin C.

According to **(Rotava et al., 2009)** the antioxidant activity of extracts from grapevine seed pressings is comparable to ascorbic acid. Due to the high content of antioxidants in the rosehip extracts, they proved high antioxidant activity in all measurements using different methods. The total antioxidant activity significantly contributed to the phenolic fraction. The comparison of the results based on the ratio of the content of the antioxidant capacity of antioxidants proved that lipophile component has been effective **(Gao et al., 2000)**. In the study, in which was evaluated the antioxidant capacity using DPPH, was found out in the red wine polyphenol content of 456 $mg.L^{-1}$ GAE **(Wang et al., 2015).** In the comparison with our results, we can state that only a part of antioxidants passes to the wine compared to the native seeds. In our experiment, the GAE concentration (grapevine seeds) was measured by method of DPPH 7.021 $g.L^{-1}$.

Table 2 Results of determination of antioxidant activity using DPPH method. Results are expressed in $mg.L^{-1}$ GAE.

	DPPH	
Grapevine seeds	7 021 ±217,7	$mg.L^{-1}$ GAE
Grapevine seed pressings	14 ±0,462	$mg.L^{-1}$ GAE
Rosehip pressings	12 ±0,35	$mg.L^{-1}$ GAE

n=6

Table 3 Results of determination of total flavonols content. Results are expressed in $mg.L^{-1}$.

	Values of total flavonols content	
Grapevine seeds	3070.0 ±116,7	$mg.L^{-1}$
Grapevine seed pressings	10.3 ±0,361	$mg.L^{-1}$
Rosehip pressings	1.1 ±0,034	$mg.L^{-1}$

n=6

Table 4 Results of determination of total hydroxycinnamic acids. Results are expressed in $mg.L^{-1}$.

	Values of hydroxycinnamic acids content	
Grapevine seeds	6 870 ±254,2	$g.L^{-1}$
Grapevine seed pressings	6.1 ±0,207	$mg.L^{-1}$
Rosehip pressings	0.9 ±0,028	$mg.L^{-1}$

n=6

Tabulka 5 Results of determination of total content of polyphenolic compounds. Results are expressed in mg/L.

	Values of polyphenolic compound content	
Grapevine seeds	7 540 ±271,4	$mg.L^{-1}$
Grapevine seed pressings	37.6 ±1,25	$mg.L^{-1}$
Rosehip pressings	3.7 ±0,13	$mg.L^{-1}$

n=6

CONCLUSION

This work deals with the study of the antimicrobial and antioxidant properties of grapevine seeds, grape pressings and rosehip pressings. The presented results highlighted the impact of grapevine seeds, grape and rosehip pressings on bacteria *C. perfringens* and *E. coli*. The highest antimicrobial effect was observed in the variants with grapevine seeds. This fact also corresponded with the content of the antioxidant components that were the highest in grapevine seeds. The values of the antioxidant activity in the grapevine seeds were much more higher than in grape pressings (500 times) and rosehip pressings (575 times higher). The grapevine seeds also contained the highest content of flavanols, hydroxycinnamic acids and the total content of polyphenolic compounds from the monitored additives.

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Acknowledgments:

Thanks for financial support to the project 9/2014 "Vliv přídavku fytogenních aditiv do krmné dávky brojlerových kuřat na jejich růst a složení mikroorganismů v trávicím traktu".

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