The impact of pseudocereal extracts with antioxidant and antimutagenic activity on development of antibiotic resistance

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Summary
This research investigates the ability of extracts from amaranth, buckwheat, Japanese millet and sorghum to influence the development of antibiotic resistance in the antibiotic-sensitive bacteria. Effects on ciprofloxacin resistance were studied in *Salmonella* Typhimurium. In case of *Staphylococccus aureus*, the study aimed at ampicillin resistance. Sorghum extract decreased the frequency of spontaneous mutations leading to both ciprofloxacin and ampicillin resistance. This extract also lowered the mutation frequency induced by 3-(5-nitro-2-furyl)acrylic acid leading to ciprofloxacin resistance. Extract from amaranth suppressed the frequency of spontaneous mutations, but increased the mutation frequency induced by 2-nitrofluoren leading to ampicillin resistance. Japanese millet, buckwheat and amaranth extracts raised the frequency of spontaneous mutations leading to ciprofloxacin resistance. Buckwheat extract displayed no effect on mutation frequency leading to ampicillin resistance. The antioxidant activity of pseudocreal extracts was also determined.

Keywords
pseudocereals; antibiotic resistance; ampicillin; ciprofloxacin; antioxidants

The emergence of microbial resistance to antibiotics represents a serious medicinal problem [1–3]. Many bacterial strains become resistant and in some cases even multiresistant to available antibiotics, which results in ineffective treatment of serious infections caused by pathogens [4]. In recent years, considerable attention is paid to the impact of diet on human health. It is known that high consumption of plant products might be associated with a reduction in the risk of chronic diseases including atherosclerosis and cancer. The beneficial effect is partly attributed to substances with antioxidant activity such as vitamins C and E, carotenoids or polyphenolic compounds [5–8].

In general, development of bacterial resistance is of genetic origin and most cases of resistance take place either via mutation or by the introduction of new genetic information [9]. Since microbial resistance primarily develops from mutations, one of the options how to prevent or suppress this unwanted phenomenon may be the use of substances with antioxidant/antimutagenic activity. Influence of antioxidants on bacteria in terms of development or inhibition of antibiotic resistance has been described. It is known that tea catechins are able to suppress development of resistance to tetracyclines, fluoroquinolones, macrolides, β-lactames and aminoglycosides [1]. The important sources of antioxidants are pseudocereals including amaranth (*Amaranthus* spp.), quinoa (*Chenopodium quinoa*), buckwheat (*Fagopyrum* spp.) and Japanese millet [10, 11]. These crops are resistant to adverse climatic conditions and they grow in regions characterized by low rainfall, drought, high temeperature or low soil fertility [12, 13]. They represent an important source of phytochemicals with nutritional and functional value [14, 15]. Buckwheat seeds contain rutin, quercetin, tocopherols and phenolic acids including *p*-hydroxybenzoic, syringic, vanilic and *p*-cumaric acid [16, 17]. On the other hand, amaranth is interesting for high content and quality of proteins, belonging to rich sources of essential amino acids such as lysine [18, 19]. Sorghum is an important source of polyphenols and plant sterols [13, 20].

The objective of this study was to determine

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the effect of DMSO (dimethylsulphoxide) extracts from buckwheat, amaranth, Japanese millet and sorghum on the mutation frequency leading to ciprofloxacin resistance in the antibiotic-sensitive strain *Salmonella* Typhimurium and ampicillin resistance in the antibiotic-sensitive strain *Staphylococccus aureus.* The antioxidant activity of the extracts, as a potential factor influencing emergence of antibiotic resistance, was also evaluated.

MATERIALS AND METHODS

Bacterial strains and antibiotics

The bacterial strains used in this study were *Salmonella enterica* subsp. *enterica* serovar Typhimurium CCM 4763 and *Staphylococcus aureus* CCM 3953, both purchased from the Czech Collection of Microorganisms (Masaryk University, Brno, Czech Republic). Antibiotics, ciprofloxacin and ampicillin were obtained from Sigma Aldrich (Steinheim, Germany).

Plant materials

Amaranth seeds, variety PI 604671, and buckwheat seeds, variety Špačinská 1, were acquired from the gene bank of the Research Institute of Plant Production (Piešťany, Slovakia). Sorghum seeds, variety Zsófia, and Japanese millet, variety Udalaja, were obtained from Plant Production Station in Uhříněves (Czech Republic).

Preparation of extracts

Extracts were prepared by alkalic hydrolysis according to KRYGIER et al. [21]. Defatted flours [22] were hydrolysed with 2 mol·l^{-1} NaOH for 4 h at 50 °C. Then, mixture was cooled, acidified with 6 mol·l^{-1} HCl to pH 2 and free polyphenolics were extracted with ethyl acetate at a ratio of 1 : 1 (v/v). Ethyl acetate extracts were evaporated to dryness in a rotary evaporator at a temperature lower than 40 °C, and the residue was dissolved in DMSO. The prepared pseudocereal extracts were kept at 5 °C.

Determination of mutation frequencies leading to antibiotic resistance

Mutation frequencies leading to ciprofloxacin and ampicillin resistance were determined as described previously [23]. Briefly, 0.5 ml of phosphate buffer (pH 7.4) and 0.1 ml of overnight culture *S. enterica* ser. Typhimurium or *Staphylococcus aureus* (cell density of 10^9 per millilitre) were pipetted into sterile tubes, to which 0.1 ml of the tested extract and, for assays on induced mutations, 0.1 ml solution of a mutagen were added, and incubated for 30 min at 37 °C. Then, 0.7 ml of Nutrient broth No. 2 (Imuna, Šarišské Michaľany, Slovakia) was added, and cultures were incubated for 3 h at 37 °C. The number of ciprofloxacin/ampicillin- resistant mutants was determined by plating the entire culture on Nutrient agar No. 2 plates containing a selective concentration of antibiotic (ciprofloxacin – 2×*MIC* (minimum inhibitory concentration) = 0.06 μg·ml^{-1}; ampicilin – 2×*MIC* = 0.250 μg·ml^{-1}). The total number of viable cells was determined by plating an appropriate dilution of the cultures on a non-selective medium. Colony forming units were counted after incubation for 24 h and, in a case of resistant colonies, on selective plates for 72 h. The influence of the extracts on mutation frequencies leading to antibiotic resistance was expressed as resistance factor (*RF*), which represented the ratio of frequency of resistant mutants affected by the extract and frequency of spontaneous resistant mutants. Mutation frequency was calculated as a mean number of resistant cells divided by the total number of viable cells per culture. A positive influence of the extract on mutation frequency leading to resistance was indicated by a decrease in *RF* compared with control (without addition of the extract). Pseudocereal extracts were tested in concentrations allowing cell viability of a minimum of 60%.

Antioxidant activity

ABTS assay

Total antioxidant activity of the extracts on ABTS (2,2'-azino-bis-[3-ethylbenthiazoline-6-sulfonic acid]) radical was determined using the method of ARTS et al. [24]. Briefly, at first the absorbance spectrum of ABTS radical cation was measured at the wavelenght from 730 to 1 100 nm. Then, 50 μl of sample was added to the cation and the absorbance spectrum was estimated after 10 min at the same wavelengths as for radical cation. The total antioxidant capacity of studied extracts was expressed as grams of Trolox ((6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) per kilogram of dry sample. The results represent a mean of two experiments.

DPPH assay

DPPH (1,1-diphenyl-2-picrylhydrazyl) radical-scavenging activity was measured according to YEN and CHEN [25]. DPPH radical was prepared by mixing 0.012 g DPPH and 100 ml 96% ethanol. For measurement of sample, 1 ml extract was pipeted into tubes, to which 3 ml ethanol and 1 ml DPPH radical were added. The absorbance of the

mixture was measured at a wavelength of 517 nm after 10 min. The antioxidant effect was also estimated as in ABTS test. The results represent a mean of two experiments.

FRAP assay

FRAP (ferric-reducing/antioxidant power) assay was carried by a method of NIEMEYER and METZLER [26] with slight modifications. For measurement, 60 μl of sample in 180 μl water was added to 1800 μl of FRAP reagent. The absorbance of the mixture was measured at a wavelength of 595 nm after 30 min. The antioxidant effect was also estimated as in ABTS and DPPH tests. The results represent a mean of two experiments.

Statistical analysis

The statistical significance of all calculated values were determined by paired Student's *t*-test. The data of antibiotic resistance represent a mean of three independent experiments. Each experiment was done in five parallel determinations. Mean values and standard errors of determination of antioxidant activity were calculated from the data obtained from two experiments.

RESULTS AND DISCUSSION

Mutations represent a primary cause of bacterial resistance. According to PILLAI et al. [1], a number of antimutagenic agents are able to suppress the emergence of resistance. However, previous results indicated only the antimutagenic potential of extracts [13, 27], but their effect on antibiotic resistance was not demonstrated.

Japanese millet extract (Fig. 1A) in lower concentrations (3.13 mg·ml^{-1} to 12.5 mg·ml^{-1}) significantly increased the spontaneous mutation frequency leading to ciprofloxacin resistance, but using higher concentrations, *RF* value did not change markedly. Buckwheat extract (Fig. 1A) slightly increased *RF*. In the highest tested extract concentration (50 mg·ml^{-1}), a 8.7-fold enhancement was observed. Extract from amaranth (Fig. 1A) influenced the mutation frequency only moderately. In the highest concentration (50 mg·ml^{-1}), the extract increased *RF* 2.7 times. On the other hand, sorghum extract (Fig. 1B) displayed slightly inhibitive effect on the frequency of spontaneous mutations. In concentration of 25 mg·ml^{-1}, the extract decreased *RF* 1.48 times and using the highest concentration of the extract, mutation frequency decreased to 62%. Differences in the effects of pseudocereal extracts on mutation frequency to ciprofloxacin resistance might be due to the differences in the contents of active compounds in the tested extracts. BÍROŠOVÁ et al. [23] found that cinnamic acid and its derivates slightly increased the mutation frequency and ciprofloxacin resistance. On the other hand, benzoic acid derivates did not display a negative influence on the mutation frequency. Only gallic acid increased the number of resistant colonies, but no cells were able to survive at selective concentrations of ciprofloxacin after the growth in an antibiotic-free medium. For that reason, authors supposed that gallic acid induced only phenotypic changes. In our previous study [13], we confirmed that derivates of cinnamic acid (ferulic, *p*-cumaric and caffeic acids) were predominant phenolic acids in the tested extracts, so they might have been responsible for the increased

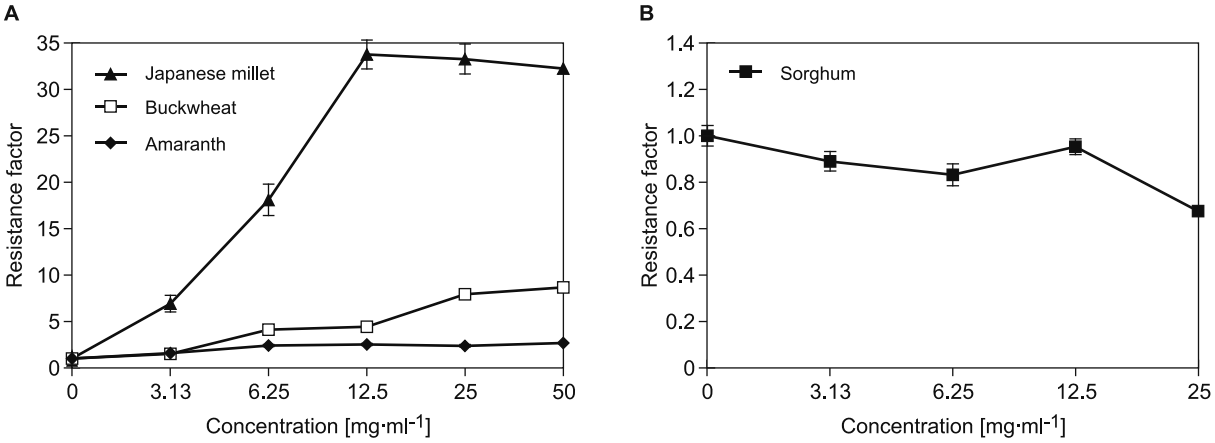

Fig. 1. Effect of amaranth, buckwheat, Japanese millet (A) and sorghum (B) extracts on the mutation frequency leading to ciprofloxacin resistance.

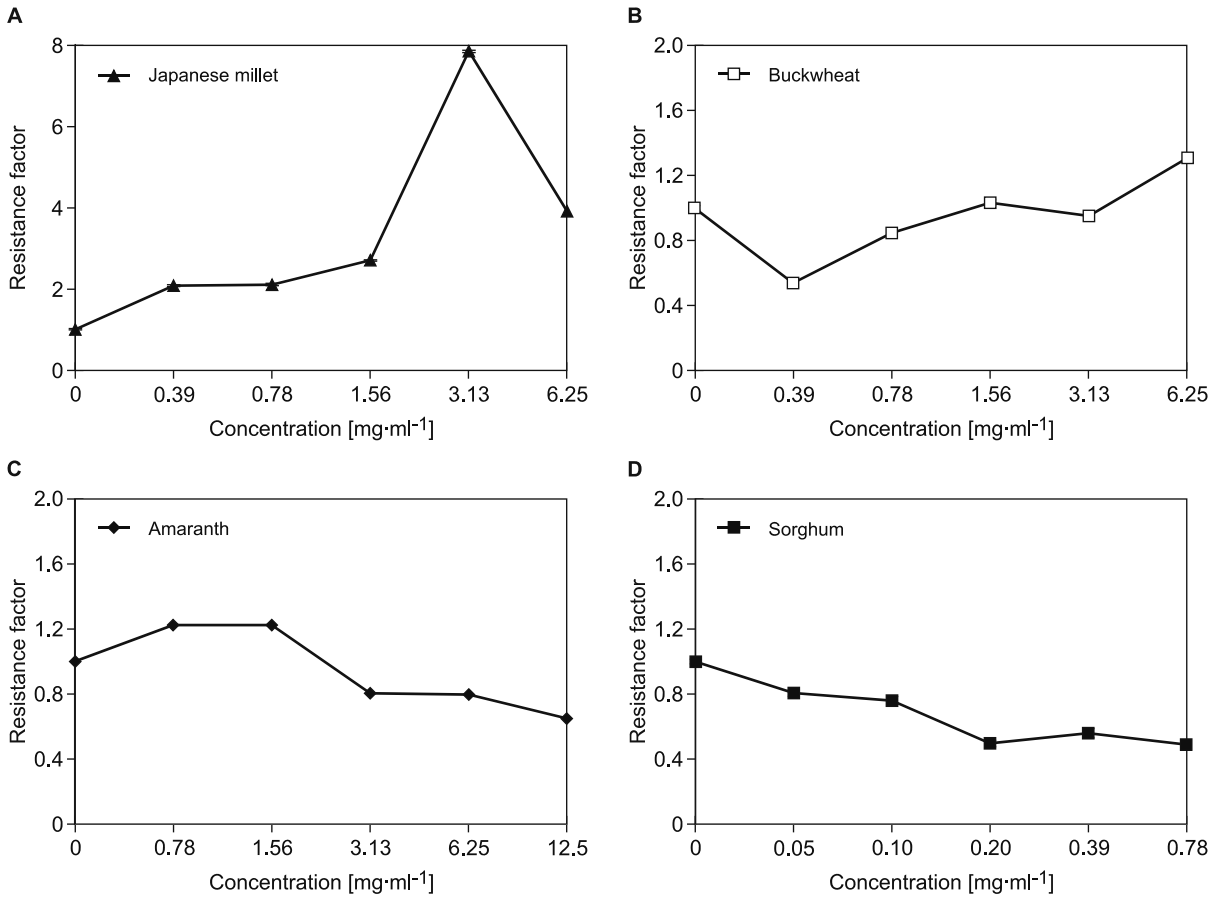

Fig. 2. Effect of pseudocereal extracts on the mutation frequency leading to ampicillin resistance.

mutation frequency and antibiotic resistance in the presence of the extracts.

In the case of ampicillin resistance, Japanese millet extract (Fig. 2A) raised the mutation frequency in the range of tested concentrations (from 0.39 mg·ml^{-1} to 6.25 mg·ml^{-1}). In the lowest concentration, the extract increased *RF* 2 times and with higher concentrations, this factor had a slightly raising trend. Buckwheat extract (Fig. 2B) moderately decreased the number of resistant colonies. This effect was also shown when *RF* was related to colony forming units. In the lowest tested concentration of the extract, a decrease by 46% in the mutation frequency was achieved. However, the use of higher concentrations of this extract did not influence the mutation frequency either in a positive or in a negative way. Conversely, the extract from amaranth did not significantly influence mutation frequency and ampicillin resistance in lower concentrations. Using higher extract concentrations, *RF* decreased and in concentration of 12 mg·ml^{-1}, a reduction by 35% of *RF* was noticed (Fig. 2C). Sorghum extract reduced the frequency of spontaneous mutations in all tested concentrations. As obvious from Fig. 2D, the tendency of reduction of ampicillin resistance was also reflected by a decrease in *RF*. In the highest concentration of the extract, a decrease by 51% of the mutation frequency and ampicillin resistance was recorded.

In the following experiments, the effects of sorghum and amaranth extracts on frequency of mutations induced by positive mutagens were studied. As a positive mutagen, 3-(5-nitro-2-furyl)acrylic acid (5NFAA) was used for determination of the ability of sorghum extract to influence the frequency of induced mutations and ciprofloxacin resistance. In the case of amaranth extract, 2-nitrofluoren (2NF) was used as a positive mutagen. The concentrations of positive mutagens (5NFAA – 3 mg·ml^{-1}; 2NF – 6 μg·ml^{-1}) were chosen to permit at least 60% cell viability. Sorghum extract significantly reduced the frequency of induced mutations leading to ciprofloxacin resistance in all tested concentrations (Fig. 3A). The reduction of mutation frequency was in the range from 21% to 70%. On the other hand, amaranth extract increased the mutation frequency (Fig. 3B). In the lowest applied concentration (0.78 mg·ml^{-1}), the extract

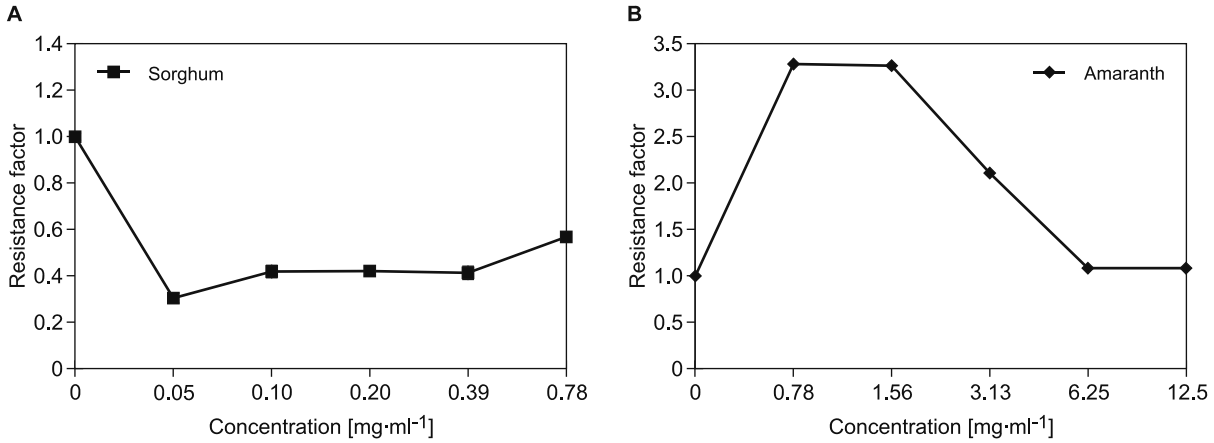

Fig. 3. Effect of sorghum and amaranth extracts on the mutation frequency leading to antibiotic resistance.
A – Effect of sorghum extract on the mutation frequency leading to ciprofloxacin resistance induced by a positive mutagen, NFAA.
B – Effect of amaranth extract on the mutation frequency leading to ampicillin resistance induced by a positive mutagen, 2NF.

raised *RF* 3.3 times, but using higher concentrations, this frequency decreased to the level of the induced mutability without the extract.

Recent research indicated the role of reactive oxygen species (ROS) in the antibiotic action [28, 29]. According to Aiassa [30], the role of ROS in the antibiotic action was related to resistance. However, the participation of antioxidant defences in the resistance to antibiotic needs to be clarified. In this context, effects like free radical scavenging might have effect on the emergence of antibiotic resistance. Therefore, this work was also aimed at the evaluation of the antioxidant activity of extracts.

As it is obvious from Tab. 1, the antioxidant capability of pseudocereal extracts measured by ABTS test was, in descending order, buckwheat = Japanese millet > sorghum = amaranth. A similar mode of antioxidant action was found by DPPH test, but statistically significant differences between samples were observed. These differences may be caused by a higher sensitivity of DPPH test. In ABTS test, the antioxidant activity value of samples actually characterizes the capability of the sample to react with $ABTS^+$ rather than to inhibit the oxidative process [31]. The best ability to reduce Fe(3+) to Fe(2+) was determined in the buckwheat extract. Our results indicate certain participation of antioxidants in the development of ciprofloxacin or ampicillin resistance. Our previous study [13] demonstrated a strong antimutagenic activity of Japanese millet extract against H_2O_2, as measured by Ames test. This fact was also confirmed by the antioxidant effect (Tab. 1). According to these results, we suggest an inhibitory effect on the development of antibiotic resistance. Surprisingly, Japanese millet extract significantly increased the frequency of spontaneous mutations to ciprofloxacin as well as to ampicillin resistance. Although the antioxidant activity of the sorghum extract was lower in comparison with other tested pseudocereals, this extract reduced the frequency of spontaneous mutations and resistance to ciprofloxacin or ampicillin. It also caused a decrease in the mutation frequency induced by NFAA leading to ciprofloxacin resistance.

A considerable attention of many scientists is devoted to emergence of antibiotic resistance. Only a limited information is available on positive or negative effects of natural compounds or foodstuffs on the development of antibiotic resistance.

Tab. 1. The antioxidant activity of extracts from amaranth, buckwheat, Japanese millet and sorghum measured by ABTS, DPPH and FRAP tests.

Sample	Antioxidant activity [g·kg^{-1}]		
	ABTS test	DPPH test	FRAP test
Amaranth	0.80 ± 0.020	3.34 ± 0.035	0.83 ± 0.037
Buckwheat	1.36 ± 0.054	5.87 ± 0.106	1.53 ± 0.077
Japanese millet	1.31 ± 0.053	4.02 ± 0.106	0.53 ± 0.024
Sorghum	0.86 ± 0.026	3.60 ± 0.021	0.41 ± 0.008

Results are means ± standard deviation (n = 2); $P < 0.05$. Results are expressed in grams of Trolox per kilogram of dry sample.

HATANO et al. [32] observed inhibitory effects of hydrolysable tannins on β-lactamase. The authors also showed that the major tea polyphenol, (–)-epigallocatechin gallate, markedly decreased *MIC* of oxacillin. On the other hand, flavonoid quercetin is a competitive inhibitor of gyrase [23]. Our results also indicated slight variations in the effects of pseudocereal extracts on the mutation frequency leading to ciprofloxacin or ampicillin resistance. These variations might be due to differences in the contents of active components and/or their synergism in the extracts. Our previous results confirmed that pseudocereal extracts contained polyphenolic compounds, flavonoids and phenolic acids [13]. The highest content of polyphenols was determined in the buckwheat extract, which contained the lowest amount of flavonoids. The polyphenol content expressed in milligrams of gallic acid per kilogram of dry sample was, in a descending order, buckwheat (2410 $mg \cdot kg^{-1}$) > Japanese millet (1323.2 $mg \cdot kg^{-1}$) > amaranth (1040.8 $mg \cdot kg^{-1}$) > sorghum (860.7 $mg \cdot kg^{-1}$). On the contrary, the sorghum extract had the highest content of flavonoids, the flavonoid content being, expressed in milligrams of rutin per kilogram of dry sample, in a descending order, sorghum (527.6 $mg \cdot kg^{-1}$) > amaranth (374.3 $mg \cdot kg^{-1}$) > Japanese millet (220.5 $mg \cdot kg^{-1}$) > buckwheat (208.3 $mg \cdot kg^{-1}$).

CONCLUSIONS

Antibiotic resistance is a major current public health problem. This research pointed out that the use of antioxidants and antimutagens is not always effective against the microbial resistance. We still do not know the mechanism of the mutual influence of the active compounds, including antimutagen – antibiotic and antimutagen – antibiotic – mutagen, respectively. For that reason, it is necessary to study these interactions and also to study their influence on the development of antibiotic resistance.

Acknowledgements

This work was supported by The Agency of the Ministry of Education, Science, Research and Sport of the Slovak Republic for the Structural Funds of Euroepan Union, Operating Program Research and Development of European Regional Developing Fund in the frame of the Project "Evaluation of natural substances and their selection for prevention and treatment of lifestyle diseases" (ITMS 26240220040), VEGA 1/0094/10 and by the Slovak Research and Development Agency in the frame of project Nr. 0310-06, and VMSP-II-0024-09. The authors thank to Výskumný ústav rastlinnej výroby Piešťany, Slovakia for providing the amaranth and buckwheat seeds and Plant Production Station in Uhříněves for providing the sorghum and Japanese millet seeds.

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Received 16 January 2012; 1st revised 28 March 2012; 2nd revised 13 April 2012; accepted 20 April 2012.