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ANTIOXIDANT ACTIVITY OF POLYPHENOLS OF ADZUKI BEAN (*VIGNA ANGULARIS*) GERMINATED IN ABIOTIC STRESS CONDITIONS*

Urszula Złotek✉, Urszula Szymanowska, Barbara Baraniak, Monika Karaś

Department of Biochemistry and Food Chemistry, University of Life Sciences in Lublin
Skromna 8, 20–704 Lublin, **Poland**

ABSTRACT

Background. Adzuki sprouts are one of more valuable but still underappreciated dietary supplements which may be considered as functional food. Sprouting reduces anti-nutritional factors and increases the bioavailability of macro and micronutrients and also affects phytochemical levels. Exposure of plants to abiotic stresses results in change in production of phytochemical compounds. The aim of this study was to assess the content and antioxidant properties of phenolic in adzuki bean seeds germinated in selected abiotic stress conditions.

Material and methods. Adzuki bean seeds were germinated in different abiotic stress conditions: thermal, osmotic and oxidative. The content of phenolics in adzuki bean seeds coat extracts and antioxidant activity Fe^{2+} chelating ability and neutralization of the free radicals generated from DPPH and ABTS were determined.

Results. All applied stress conditions (except for thermal stress) have caused decrease the content of the analysed phenolic fractions. The lowest amounts of polyphenols in extracts of sprouts obtained in oxidative stress conditions were observed. The highest ability to neutralize free radicals generated with ABTS and DPPH have extracts from sprouts germinated under thermal stress 39.94 and 13.20 μmol TEAC/g d.w., respectively. The lowest – sprouts obtained in oxidative stress conditions (18.2 and 9.72 μmol TEAC/g d.w.). The highest ability to chelate Fe^{2+} has been shown by the extract from adzuki bean seeds coat subjected to thermal stress (7.06 %) and the lowest control extract (3.08%).

Conclusions. It can be concluded that only thermal stress contributes to the improvement of antioxidant activity of extracts obtained from adzuki bean seeds coat.

Key words: adzuki bean sprouts, abiotic stress, antioxidant activity, polyphenols

INTRODUCTION

Food legumes have been playing a very important role in the human diet for a long time. In recent years consumers' interest in healthy food has increased. Dieticians recommend legume plants which are a valuable source of proteins, but also vitamins, minerals and various bioactive components (Tharanathan and Mahadevamma, 2003). An increasingly popular addition to dishes are sprouts plants from Far East, including

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✉urszula.zlotek@up.lublin.pl, phone +48 81 462 3328, fax +48 81 462 3324

adzuki beans. The sprouts are excellent examples of 'functional food' defined as lowering the risk of various diseases (Bouis, 2003; Randhir et al., 2004). Legume sprouts are rich in amino acids, macro- and microelements (iron, magnesium, potassium, sulfur, iodine, manganese, lithium, selenium), and a variety of vitamins, including A, B, C, E, H, K, PP. They are recommended for people with ailments of the pancreas and kidneys (Li et al., 2011), but the beneficial effects are especially attributed to phytochemicals such as phenolic compounds. During the germination of the seeds, nutrients under influence of moisture, temperature, and primarily enzymes action are transformed into compounds easily and quickly absorbed by the body. The high content of bioactive compounds causes that consumption of sprouts strengthens body's immunity.

All environmental factors disrupting plant growth by interfering with the metabolic processes of cells are determined stress factors. These factors may be abiotic (UV radiation, temperature, salinity) and biotic (plants, animals, micro-organisms). First of all the effect of stress on the plant is manifested by a decrease in the photosynthetic activity of plants. A reduction in the amount of photosynthetic surface, a faster decay of photosynthetic pigments, disruption of stomatal activity and adverse changes in the intensity of gas exchange occur (Matysiak and Adamczewski, 2009). Various abiotic stresses may contribute to the biosynthesis and accumulation of secondary metabolites, including polyphenolic compounds which have a variety of effects, including antioxidant, antimicrobial, antiviral, anti-allergic and anti-inflammatory actions (Ramakrishna and Ravishankar, 2011).

Exposure of plants to abiotic stresses results in an increased production of reactive oxygen species (ROS), which cause damage to cell components (Nagesh Babu and Devaraj, 2008). Temperature is a very important factor which significantly affects the efficiency of sprouting. Salinity is one of the major physical parameters of an environment, which affects almost every aspect of physiology and biochemistry of plants. The high exogenous salt concentration influences on seed germination, water deficit, causes electrolyte imbalance in the cell and leads to osmotic stress (Kaymakanova and Stoeva, 2008). Oxidative stress is the result of an imbalance between production and neutralization of free radicals. Among the oxygen free radicals an important one is superoxide anion ($O_2^{\bullet-}$) and its conversion products, such as hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet-}$) and peroxynitrite ($ONOO^-$). Hydrogen peroxide is more stable than other ROS, readily diffuses across cell membranes and is often used as a model to test the vulnerability of plants to oxidative stress (Quan et al., 2008).

The aim of this work was to study the effects of selected types of stress on the content of phenolic compounds in adzuki bean seeds coat and their antioxidant properties.

MATERIAL AND METHODS

Plant material and growth conditions

Dry adzuki bean (*Vigna angularis*) were purchased from W. Legutko Breeding and Seed Company. Been seeds were washed few times with distilled water to remove contaminants and left in water for 5 hours at room temperature. They were germinated in Petri dishes (65 mm) containing a sheet of filter paper (Whatman No. 1), moistened with distilled water. Germination was conducted in incubator (+25°C in darkness), samples during cultivation of sprouts were wetted with distilled water twice a day.

After two days of culture they were subjected to appropriate stress conditions:

- thermal stress – two hours heating in 40°C (T)
- osmotic stress – sprouts watered within 24 hours with 200 mM NaCl (NaCl)
- oxidative stress – sprouts watered within 24 hours with 200 mM H_2O_2 (H_2O_2)
- control (without stressor) (C).

After 5 days of culture, sprouts were harvested and frozen in order to inhibit biochemical processes. Then seed beans were shelled, lyophilized and then ground in a mill husk laboratory and stored at 4°C.

Extraction procedure

Lyophilized seeds coat (3 g in triplicate) was homogenized with 10 ml of acidified methanol (0.1% HCl) and the phenolics were extracted for 1 h at 4°C, then centrifuged at 10 000 g for 30 min – this procedure was repeated five times and the supernatants were combined and added to 50 ml – it was a crude extract of polyphenols. In crude extracts the content of total phenolics, total anthocyanins, flavonoids, phenolic acids and antioxidant activities was determined.

Total phenolic content. The analysis was carried out with the Folin-Ciocalteu's method (Singleton et al., 1974). 0.1 mL of each extract was pipetted into different test tubes. To this solution 0.1 ml of distilled water, and 0.4 ml of Folin-Ciocalteau reagent (diluted with distilled water 1:5) were added. After three minutes 2 ml of sodium carbonate solution (10%) were added and vigorously mixed. After 30 minutes the absorbance was measured at 725 nm using a Lambda 40 UV-Vis spectrophotometer. The total phenolic content was expressed as gallic acid equivalent through the calibration curve of gallic acid.

Total anthocyanin content. The total anthocyanin content was determined using the pH differential method (Giusti and Worlstad, 2001). Two dilutions of the same sample were prepared in 0.025 M potassium chloride solution and in 0.4 M sodium acetate solution adjusted to pH 1.0 and 4.5 with HCl, respectively. The absorbance of each dilution was measured at 520 and 700 nm against a distilled water blank using a Lambda 40 UV-Vis spectrophotometer. Absorbance (A) was calculated as follows:

$$A = (A_{520} - A_{700})_{pH1.0} - (A_{520} - A_{700})_{pH4.5}$$

The anthocyanin concentration in the original sample was calculated using the following formula:

$$\text{Anthocyanin content (mg/L)} = \\ = (A \cdot MW \cdot DF \cdot 1000)/(\varepsilon \cdot 1)$$

where: MW – the molecular weight of cyanidin-3-O-glucoside (449.2 g·mol^{-1}), DF – the dilution factor, and ε – the molar extinction coefficient of cyanidin-3-glucoside (ε = 26 900 L·cm^{-1}·mol^{-1}).

Flavonoid content (Lamaison and Carnet, 1990). Total flavonoid content was measured by aluminium chloride colorimetric assay. To 0.5 ml of acidified methanolic extract 0.5 ml of 2% solution of $AlCl_3 \cdot 6\ H_2O$ in methanol was added. The absorbance was measured after 10 minutes against methanol at 430 nm. Flavonoid concentration was expressed in mg/g dry weight (mg/g d.w.) as quercetin equivalent.

Phenolic acids content. The total content of phenolic acids was marked of spectrometric Arnova method (Szaufer-Hajdrych, 2004) and expressed as caffeic acid equivalents. Mixed: 0.6 ml of water, 0.1 ml of extract, 0.1 ml of 0.5% hydrochloric acid, 0.1 ml of Arnov reagent (10 g of sodium molybdate and 10 g of sodium nitrate dissolved in 100 ml of methanol) and 0.1 ml of 1 M NaOH. The absorbance was measured at 490 nm against blank sample containing methanol instead of extract.

Antioxidant properties

Free radical scavenging assay. Free radical scavenging activity was measured using 1,1-diphenyl-2-picrylhydrazyl (DPPH) – according to Brand-Williams et al. (1995) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) – according to Re et al. (1999) – as the source of the free radicals. For the DPPH assay, 0.08 mL of extracts was mixed with 1.92 mL 6·10^{-5} M solution of DPPH$^{\bullet}$ in methanol. Absorbance was measured at λ = 515 nm both immediately and after 2.5 min of incubation against a blank sample containing methanol.

For ABTS assay, ABTS radical cations (ABTS$^{+\cdot}$) were produced by reacting 7 mM stock solution of ABTS with 2.45 mM potassium persulphate (final concentration) and allowing the mixture to stand in the dark for at least 6 h at room temperature prior to use. The ABTS$^{+\bullet}$ solution was diluted to an absorbance of 0.7 ±0.05 at 734 nm (Lambda 40 UV-Vis spectrophotometer, Perkin Elmer). A 0.04 mL aliquot of extract was added to 1.8 mL of ABTS$^{+\bullet}$. The absorbance at 734 nm was measured both at the beginning and after 2.5 min of reaction against a blank sample containing 1.8 mL of water and 0.04 mL of methanol. The ability of test material to quench DPPH or ABTS free radicals was evaluated according to the equation:

$$\text{scavenging \%} = [(A_C - A_A) / A_C] \cdot 100$$

where: A_C – absorbance of control (solvent instead extract), at 0 min, A_A – absorbance of sample after 2.5 min.

The antioxidant activity was related to Trolox (an analogue of vitamin E) and expressed as µmol of Trolox per gram of dry weight (DW; TEAC, Trolox equivalent antioxidant activity). The standard curve was prepared in the concentration range 0–1500 µmol of Trolox (r^2 = 0.978) for DPPH assay and 0–800 µmol of Trolox (r^2 = 0.985) for ABTS assay.

Chelating power was determined using the method of Guo et al. (2001). The extract samples (1 ml) were added to 0.02 mL of 2 mM $FeCl_2$ solution and 0.04 mL 5 mM ferrozine and the mixture was shaken vigorously and left standing at room temperature for 10 min. Then, absorbance of the solution was measured spectrophotometrically at 562 nm against a blank sample (methanol). The control sample contained 1 mL of methanol, 0.02 mL 2 mM $FeCl_2$ solution and 0.04 mL 5 mM ferrozine. The percentage of inhibition of ferrozine – Fe^{2+} complex formation was calculated using the formula:

$$\% \text{ inhibition} = (1 - A_A/A_C) \cdot 100$$

where: A_C – absorbance of control (solvent instead extract), A_A – absorbance of sample.

Statistical analysis

All experimental results were means and were performed in triplicate (three extracts and three measurements for each extract) and the data in the tables and figures represent mean values ±standard deviation ($n = 9$). Results were evaluated for statistical significance using univariate analysis of variance (ANOVA) with Statistica 6.0 software (StatSoft, Inc., Tulsa, OK, USA) and Tukey's post hoc test. The factor was elicitor type. Differences were considered significant at $p < 0.05$.

RESULTS

All the applied stress factors caused a decrease in the content of total phenolic compounds in adzuki beans seeds coat. The lowest amount both total phenols and phenolic acids (almost three fold lower than in control) was observed in case of seeds germinated in oxidative stress conditions (H_2O_2). The content of flavonoids also decreased due to applied NaCl and H_2O_2 as stressors, but increase was observed when elevated temperature was used. The same situation was noted in case of anthocyanins (Table 1).

In this research the antioxidant activity of adzuki been seeds germinated in stress conditions was also determined. Antiradical activity designated against $ABTS^{+\bullet}$ was similar for control and thermally stressed seeds extracts: 39.00 and 39.94 TEAC μmol·g^{-1} d.w., respectively. Significantly lower antioxidant activity in comparison to control showed extracts from adzuki bean seeds sprouted in osmotic and oxidative stress conditions (18.20 and 20.83 μmol·g^{-1} d.w., respectively; Fig. 1).

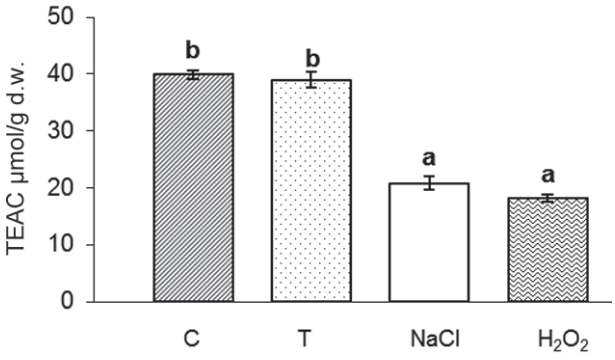

Fig. 1. Antiradical activity of extracts obtained from adzuki bean seeds coat determined against $ABTS^{+\bullet}$. Results are means ±SD of three independent measurements. Values with different letters superscripts are significantly different at α = 0.05

Table 1. Effect of abiotic stresses on contents of phenolic compounds (mg/g d.w.) in crude extracts from adzuki seeds coat

Stress conditions	Content, mg/g d.w.			
	total polyphenols	anthocyanins	flavonoids	phenolic acids
Control (C)	22.767 ±0.555a	0.019 ±0.0018a	0.688 ±0.011b	0.019 ±0.001b
Thermal (T)	21.778 ±0.221a	0.083 ±0.015b	0.702 ±0.02b	0.017 ±0.003b
Osmotic (NaCl)	8.824 ±0.297c	0.005 ±0.011a	0.375 ±0.011a	0.008 ±0.002a
Oxidative (H_2O_2)	7.763 ±0.424b	0.004 ±0.0a	0.360 ±0.002a	0.006 ±0.0009a

Mean ±standard deviation. Statistically significant differences ($p < 0.05$) indicated various letters.

Similar trend was observed for DPPH method (Fig. 2). The highest ability of neutralizing free radicals generated from DPPH was noted for adzuki bean seeds germinated in thermal stress conditions (13.20 TEAC μmol·g^{-1} d.w.). While the lowest was determined for extracts obtained from adzuki been seeds coat after using H_2O_2 as a stressor (9.72 TEAC μmol·g^{-1} d.w.).

In Figure 3 chelating properties of adzuki bean seeds coat extracts relative to Fe (II) were shown. The highest ability to chelate iron ions was shown by extracts obtained from adzuki been seeds germinated in thermal stress conditions (7.06%), while the lowest control extracts (3.08%). Higher chelating properties in relation to control was also shown by phenolics isolated from adzuki bean sprouted in osmotic and oxidative stress conditions (5.64% and 6.32%, respectively).

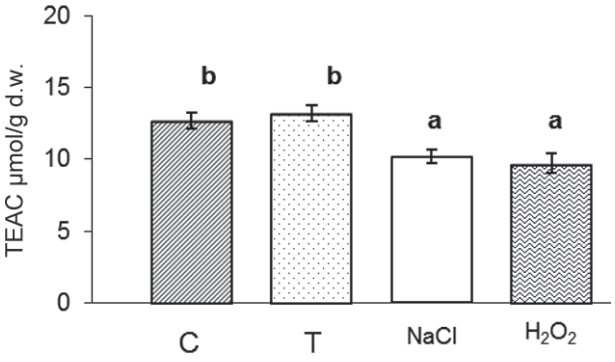

Fig. 2. Antiradical activity (TEAC μmol/g d.w.) of adzuki bean seeds coat extracts determined against DPPH•. Results are means SD of three independent measurements. Values with different letters superscripts are significantly different at α = 0.05

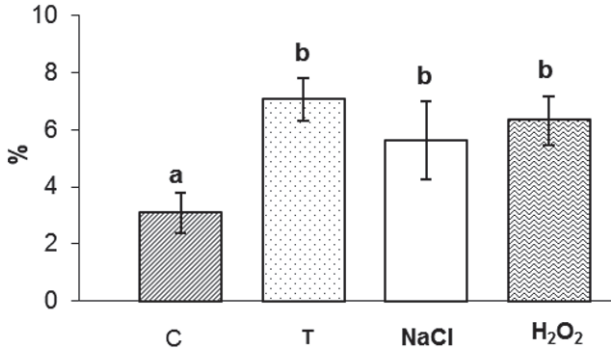

Fig. 3. Fe^{2+}chelating ability (%) of adzuki bean seeds coat extracts germinated in abiotic stress conditions. Results are means SD of three independent measurements. Values with different letters superscripts are significantly different at α = 0.05

DISCUSSION

In recent years sprouts has become increasingly popular in human diet because they are a rich source of many nutritive and bioactive compounds. Germination is a technology that involves physiological changes that improve the digestibility and nutritive value of legumes (Urbano et al., 2005), as well as may be a strategy to increase the content of some bioactive compounds (Fernandez-Orozco et al., 2008; Mwikya et al., 2001). Only few studies involving adzuki sprouts related to levels of bioactive compounds have been conducted so far. High levels of phenolic compounds, including flavonoids, measured in the seed, are attributed to their strongly coloured maroon seed coats. These mainly contain proanthocyanins (Ariga et al., 1988; Ariga and Hamano, 1990).

Besides germination, some researches are still looking for new, effective methods of improving the quality of plant food. Elicitation has been used to induce production of many bioactive compounds *in vitro* plant cell cultures (Ramakrishna and Ravishankar, 2011), as well as in the whole edible plants (Złotek et al., 2014; Szymanowska et al., 2015). Germination supported by elicitation has also been studied as a method of improving the quality (especially connected with the content and activity of phytochemicals) of sprouts (Świeca et al., 2014; Randhir et al., 2004; Limón et al., 2014). For example Randhir et al. (2004) have demonstrated that elicitation with natural elicitors such as fish protein hydrolysates, lactoferrin and oregano extract significantly improved the phenolic, antioxidant and antimicrobial properties of mung bean sprouts. Also, in the study delivered by Oh and Rajashekar (2009) the use of environmental shocks like high light and chilling increased the total phenolic content which was correlated with higher antioxidant activity in alfalfa, broccoli and radish sprouts. In the present study the use of elevated temperatures (40°C) during germination also caused an increase in the content of anthocyanins

and flavonoids in the tested sprouts and did not significantly alter the content of total phenolic compounds and phenolic acid (Table 1). Similarly, Świeca et al. (2014) demonstrated that temperature stress (both low and high) may cause increasing of polyphenols content in fresh lentil sprouts.

However, in some cases the content of phenolic compounds may be reduced during germination under stress conditions. This is confirmed by Weidner et al. (2007). These researchers germinated *Vitis amurensis* seed under osmotic stress conditions. The content of phenolic compounds was reduced in relation to the seeds germinated under optimal conditions. Similarly, used in the present study abiotic elicitation with osmotic and oxidative stress caused a decrease in the content of phenolic compounds in adzuki bean seeds coat (Table 1). Some researchers suggested that salt stress creates in plants ionic and osmotic stress resulting in accumulation or decrease of some secondary metabolites in plants – the effect depends, to some extent, on the sensitivity of the plant to this type of stress conditions (Ramakrishna and Ravishankar, 2011; Mahajan and Tuteja, 2005).

There are also some reports concerning the elevation of the antioxidant potential of edible sprouts by elicitation (Świeca et al., 2012; Khattak et al., 2007; Gawlik-Dziki et al., 2013). Obtained in the present study result indicated that phenolic compounds concentration in adzuki bean seeds coat was only partially correlated with the antioxidant activity of extracts. Elicitation with tested factors caused statistically significantly increasing only chelating ability of adzuki bean sprouts, whereby the largest increase was achieved after elicitation with high temperature (Fig. 3). The poor correlation between content of polyphenols and antioxidant activity of adzuki bean sprouts demonstrated in this study may be due to different phenolic composition similarly to Limón et al. (2014) research. There are some reports which confirmed this observation (Shivashankara and Acharya, 2010; Gawlik-Dziki and Świeca, 2011), because the antioxidant activity of phenolics is related to their chemical structure. The results obtained by Świeca and Baraniak (2014) indicated that elicitation with H_2O_2 may influence the quantitative-qualitative profile of phenolic compounds in lentil sprouts which can be determined by the biological activity of sprouts. In addition, Burguieres et al. (2007) suggested that some polyphenols do not act as antioxidants because some of them are involved in lignification and structural development during germination and growth processes.

CONCLUSIONS

Our research has demonstrated that the specific factors used in the cultivation of sprouts can induce or reduce the synthesis of particular physiologically active ingredients and modify their activity. The best factor for elicitation of adzuki bean sprouts was high temperature. So, under certain conditions you can get food to achieve the targeted effects on the human body.

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WŁAŚCIWOŚCI PRZECIWUTLENIAJĄCE ZWIĄZKÓW FENOLOWYCH FASOLI ADZUKI (*VIGNA ANGULARIS*) PODDANEJ KIEŁKOWANIU W WARUNKACH STRESÓW ABIOTYCZNYCH

STRESZCZENIE

Wstęp. Kiełki fasoli adzuki są jednym z bardziej wartościowych, ale niedocenianych suplementów diety, które mogą być uznane za żywność funkcjonalną. Kiełkowanie zmniejsza ilość substancji antyżywieniowych i zwiększa biodostępność makro- i mikroelementów oraz wpływa na poziom związków bioaktywnych. Ekspozycja roślin na stresy abiotyczne powoduje zmiany w produkcji związków fitochemicznych. Celem pracy była ocena zawartości i właściwości przeciwutleniających związków fenolowych fasoli adzuki kiełkowanej w warunkach wybranych stresów abiotycznych.

Materiał i metody. Nasiona fasoli adzuki kiełkowano w warunkach stresów: termicznego, osmotycznego i oksydacyjnego. W ekstraktach otrzymanych z okrywy nasiennej kiełków fasoli adzuki oznaczono zawartość związków fenolowych i właściwości przeciwrodnikowe wobec $DPPH^{\bullet}$ i $ABTS^{\bullet+}$ oraz zdolność do chelatowania jonów Fe (II).

Wyniki. Wszystkie zastosowane warunki stresowe (oprócz stresu termicznego) wpłynęły na zmniejszenie zawartości analizowanych frakcji fenolowych. Najmniejszą zawartość polifenoli zaobserwowano w ekstraktach uzyskanych w warunkach stresu oksydacyjnego. Największą zdolność do neutralizacji wolnych rodników generowanych z ABTS i DPPH wykazały ekstrakty z nasion kiełkujących w warunkach stresu termicznego, odpowiednio 39,94 i 13,20 μmol TEAC/g s.m. Najmniejszą zdolnością charakteryzowały się kiełki uzyskane w warunkach stresu oksydacyjnego (18,2 i 9,72 μmol TEAC/g s.m.). Największą zdolność chelatowania Fe (II) wykazały ekstrakty z okrywy nasion fasoli adzuki kiełkowanej w warunkach stresu termicznego (7,06%), a najmniejszą – ekstrakt kontrolny (3,08%).

Wnioski. Można stwierdzić, że tylko stres termiczny przyczynił się do poprawy aktywności przeciwutleniającej ekstraktów uzyskanych z okrywy nasiennej fasoli adzuki.

Słowa kluczowe: kiełki fasoli adzuki, stres abiotyczny, właściwości przeciwutleniające, polifenole

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