

Full Length Research Paper

Antioxidant activity and total phenolic content of earthworm paste of *Lumbricus rubellus* (red worm) and *Eudrilus eugenia* (African night crawler)

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Earthworm paste (EP) has many therapeutic properties such as antioxidant, anti-inflammatory, anticancer and antibacterial activities. Antioxidant properties of the earthworm paste extract may attribute to high phenolic compounds. This study involved quantification of phenolic compounds and evaluation of the antioxidant activity of earthworm paste (EP) from *Lumbricus rubellus* (red worm) and *Eudrilus eugeniae* (African night crawler). Three types of solvents were used: 75% methanol, 80% methanol and 80% ethanol. Total phenolic compounds were quantified spectrophotometrically using Folin-Ciocalteu reagent. The antioxidant activity of earthworms paste was evaluated using DPPH (1,1-diphenyl-1-2-picrylhydrazyl) method. The extraction of phenolics with 80% ethanol in both species yielded the highest amount of phenolics, 220.6 mg/L, in the African night crawler extract and 247.00 mg/L in the red worm extract. Extraction with 80% methanol yielded 217.00 mg/L of phenolics in the African night crawler extract and 228.00 mg/L in the red worm extract. Extraction with 75% methanol yielded higher amounts in red worm (237.00 mg/L) than in African night crawler (207.00 mg/L). EP of the African night crawler yielded a greater amount of phenolic compounds than the red worm. DPPH assay showed that the inhibition activity of African night crawler (95.23%) was significantly higher than that of the red worm extract (38.26%) in 80% methanol, whereas, in 80% ethanol, the inhibition activity of African night crawler (48.13%) was not significantly different from that of the red worm extract (47.73%). Inhibition of antioxidant activity of African night crawler extract in 75% methanol (48.13%) was higher than that of the red worm extract (47.93%). The study showed that earthworm paste has notable amounts of phenolic compounds that have significant antioxidant activity.

Key words: Earthworm paste (EP), phenolic compounds, antioxidants.

INTRODUCTION

Earthworms are invertebrate animals that have a tube-shape, soft and segmented body. They belong to Oligochaeta of the Annelida phylum. For thousands of years, earthworms have been used for their therapeutic benefits (Ranganathan, 2006). They also have many bioactive compounds that were extracted and purified by native

peoples in many different countries (Zhao et al., 2005). Recently, earthworms have been the subject of a widespread investigation because they have unique therapeutic properties, including anti-inflammatory, anti-oxidative, anti-tumor and anti-bacterial effect (Balamurugan et al., 2007; Li et al., 2011).

Phenolics are secondary metabolites and act as free radical scavenging molecules (Katalinic et al., 2010). Reactive oxygen species (ROS) cause many severe ailments such as cancer, and also accelerate aging (Dai and Mumper, 2010). Antioxidants, on the other hand, play a vital role in preventing body cell damage caused by the activity of radicals and other oxygen species inside the human body (Zheng and Wang, 2001).

Antioxidant activity is directly attributed to phenolic compounds that play a vital role in neutralizing the free radicals because phenolics have a hydroxyl group (Balamurugan et al., 2007). Limited studies have been done on the extraction of phenolic compounds from the earthworm species *Lumbricus rubellus* (red worm) and *Eudrilus eugeniae* (African night crawler). Most of the earlier studies focused primarily on the activity of different biologically active compounds such as glycolipoprotein. Some of these studies assessed the activity of glycolipoprotein molecules (G-90) (Grdisa et al., 2001; Balamurugan et al., 2008). Other studies evaluated the activity of other biological active compounds such as polysaccharides, peptides (Liu et al., 2004) and fibrinolytic enzymes (Cheng et al., 2008; Nakajima et al., 2003).

Therefore, the aim of the present study was to quantify the phenolic contents and to evaluate the antioxidant activity of earthworm paste of red worm and African night crawler.

MATERIALS AND METHODS

The procedure detailed in Prakash et al. (2007) was adopted for extraction of earthworm paste with modifications. Earthworm *L. rubellus* and *E. Eugenia* were obtained from the GNR enterprise in Malaysia. 1.5 kg of mature earthworms for each species were washed with tap water and left in a tray containing paper for gut clearance for 10 h. The worms were then washed with distilled water and kept in a plastic tray. Finally, the worms were exposed to the sun for two days to kill them. The coelomic fluid that leaked from the worms was collected.

Extraction procedure

The brown-coloured samples of earthworm paste (EP) were centrifuged at 4000 rpm for 20 min to separate the liquid from other particles. One milliliter of the liquid was added to each of four test tubes, and then 5 ml of different solvent compounds: 75% methanol, 80% methanol, 75% ethanol and 80% ethanol were added to the mixture. The mixture was sonicated for two hours and incubated at room temperature for 24 h.

Quantification of phenolic compounds contents

The samples were subjected to the Folin-Ciocalteu reagent procedure to determine the total phenolic contents of the earthworm paste. One hundred microliters of the sample was extracted from each group of earthworms and mixed in a test tube with 200 μ L deionized water. Two hundred microliters of Folin-Ciocalteu reagent were added to the same tube. Finally, after 3 min, 1000 μ L of sodium carbonate (Na_2CO_3) was added to the mixture. The samples were

incubated at room temperature for two hours and shaken for two hours until homogenised. Gallic acid was used as the standard and water was used as the blank. The concentration of the total phenolics in the mixture was read at a wavelength of 765 nm using a spectrophotometer. The calibration curve was obtained by plotting five gallic acid concentrations (10, 40, 50, 75 and 100 mg/L). The reading of absorbance at 765 nm was done by using the spectrophotometer. The total phenolic content (TPC) was expressed as microgram gallic acid equivalent per gram of sample. The experiment was done in triplicate.

Antioxidant activity

The antioxidant activity was adopted from Alam et al. (2010) with slight modification. Different concentrations of the samples of earthworm paste of red worm and African night crawler were pre-prepared in methanol in five concentration (40, 80, 120, 160, 180 and 200 ppm). DPPH solution was prepared in methanol by adding 0.0025 g in order to get the final concentration of DPPH 25 ppm. 0.5 ml of each concentration in methanol was added to 3.5 ml of DPPH solution. The mixture was shaken and incubated for 30 min in the dark at room temperature. The absorbance was read at 517 nm by using the spectrophotometer. The control sample was pre-prepared by following the same method without the sample. Ascorbic acid was used as positive control standard. Inhibition of DPPH free radical in percent was evaluated according to the formula: Inhibition (%) = $[(A_{\text{control}} - A_{\text{sample}})/A_{\text{control}}] \times 100$, where A_{control} represents the absorbance of the control reaction (containing all reagents except the test compounds), A_{sample} represents the absorbance of test samples. L-ascorbic acid was used as positive control.

Statistical analysis

Two-way ANOVA was used to test the differences in means of total phenolic content between the earthworm species and (TPC). Independent sample t-test was used to compare the mean values of total phenolic content and antioxidant activity between red worm and African night crawler worms. The data were expressed as mean \pm standard deviation.

RESULTS

Quantification of phenolic compounds using the Folin-Ciocalteu method

Statistical analysis showed that no significant differences between types of organic solvents were observed. It was also indicated that differences between the amounts of phenolics in red worm extracts and African night crawler extracts was significance (Figure 1). Moreover, statistical analysis showed the differences of amounts of phenolic compounds among different types of solvents. Extraction with 80% ethanol in red worm and African night crawler produced the highest amounts: 247 and 207 mg/L, respectively. Extraction with 80% methanol in red worm yielded 228 mg/L as compared to African night crawler extract which yielded 217 mg/L. The quantity of phenolics obtained in 75% methanol extraction in red worm and African night crawler gave 237 and 207 mg/L, correspondingly (Table 1).

Furthermore, the results showed that extraction with 80% ethanol in the red worm and the African night crawler

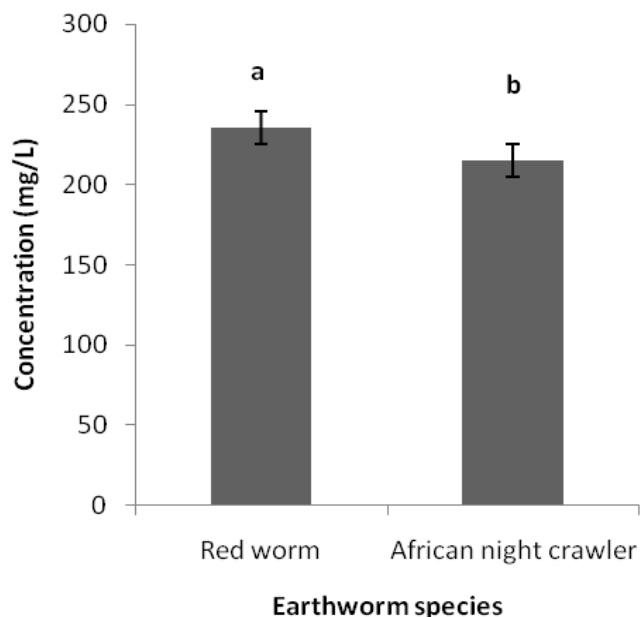


Figure 1. Average of total phenolic contents of earthworm paste of red worm and African night crawler as influenced by different solvents. Bars represent standard error differences of means (\pm SEM). Mean values followed by the same letter are not significantly different at $p \leq 0.05$.

Table 1. Total phenolic compound contents in earthworm paste of red worm (*Lumbricus rubellus*) and African night crawler (*Eudrilus eugeniae*) in different solvent types.

Earthworm specie	Solvent	TPC ^a
Red worm	80% Methanol	228.60 \pm 13.07
Red worm	75% Methanol	237.30 \pm 9.53
Red worm	80% Ethanol	247.30 \pm 16.40
African night crawler	80% methanol	217.00 \pm 2.60
African night crawler	75% Methanol	207.00 \pm 5.50
African night crawler	80% Ethanol	220.60 \pm 16.40

a TPC :Total phenolic content (mg/L), the results were expressed as mean \pm standard deviation for three replicates.

produced the highest amounts of phenolic contents: 247 and 220 mg/L, respectively. Extraction with 80% methanol in the red worm yielded 228 mg/L, whereas African night crawlers extract yielded 217 mg/L. The quantity of phenolics obtained in 75% methanol extraction in the red worm and the African night crawler extract gave 237 and 207 mg/L, respectively (Table 1).

The inhibitory activity of extract using the DPPH method

The comparison between antioxidant activity of red worm and the African night crawler extract is shown in Table 2.

Table 2. Inhibition activity of earthworm paste and ascorbic acid.

Sample	Inhibition (%)
Red worm in 80% methanol	38.26 \pm 0.35
Red worm in 80% ethanol	47.73 \pm 0.15
Red worm in 75% methanol	47.93 \pm 0.05
African night crawler in 80% methanol	59.23 \pm 0.15
African night crawler in 80% ethanol	48.13 \pm 0.57
African night crawler in 75% methanol	56.40 \pm 0.34
L-ascorbic acid	95.60 \pm 2.50

Values are expressed as a mean \pm standard deviation ($n = 3$).

The inhibitory activity of African night crawler extract (59.23%) in 80% methanol was higher than that of the red worm extract (38.26%), whereas, in extraction with 80% ethanol, the inhibitory activity of African night crawler (48.13%) was similar to that of the red worm extract (47.73%). Inhibition of antioxidant activity of African night crawler extract in 75% methanol (48.13%) was stronger than that of the red worm extract (47.93%). The inhibitory activity of L-ascorbic acid was 95.6%. The results revealed that African night crawler worm extract had the strongest inhibition activity than the red worm extract (Figure 2).

DISCUSSION

Ethanol can effectively extract phenolic compounds from their natural sources and the solubility of phenolic compounds can be increased using a mixed solvent (Spigno et al., 2007). Changes in ethanol concentration can also modify the solubility of phenolic compounds, and this may influence the extraction process (Cacace and Mazza, 2003). This finding is consistent with the results reported by Turkmen et al. (2007). They found that the highest extraction yields for the extraction of phenolic compounds such as rosmarinic and caffeic acids from some herbs was between 30 and 60%. Similar results were obtained by Pinelo et al. (2004) who found that the ethanol extraction had a higher content of phenolics in almond hull.

Using methanol as an extraction agent has been considered the most effective procedure for the extraction of phenolic compounds. It has also been shown to be highly effective in extraction of low molecular weight polyphenols (Kapasakalidis et al., 2006). Apart from methanol concentration in water, different parameters have an effect on the extraction of phenolic compounds such as particle size and number of extraction stage and the presence of interfering substances (Nepote et al., 2005). This is consistent with results by Castaneda-Ovando et al. (2009) who stated that extraction of phenolic compounds (anthocyanins) from grape pulp using methanol was 20% more productive than that with ethanol.

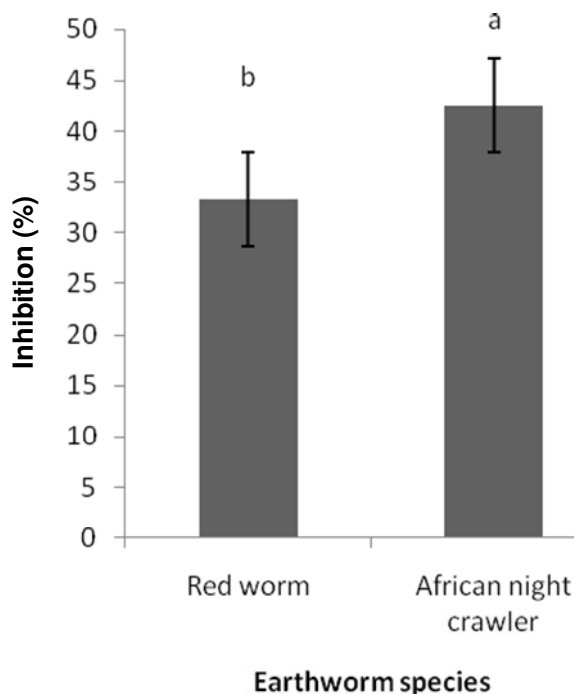


Figure 2. Comparison of inhibition activity of earthworm paste of red worm and African night crawler. Bars represent standard error differences of means (\pm SEM). Means values followed by the same letter are not significantly different at $p \leq 0.05$.

The results also indicated that differences between the amounts of phenolics in the red worm extracts and the African night crawler extracts were significant ($p \leq 0.05$). This might be because of different factors that affect the biology of earthworms. For example, Ranganathan (2006) reported that these different factors were physical, chemical and biological properties of soil, types of plant litters, animal's dung and type of microorganisms and nematodes.

Methanolic extraction of the earthworm paste of the red worm and the African night crawler exhibited the strongest antioxidant activity. The inhibitory activity of the African night crawler extract in 80% methanol was 59.23%, while inhibitory activity of red worm extract in 75% methanol was 47.93%. These results show the fact that aqueous methanol was more effective than other solvents. Methanol has hydroxyl group which may form intramolecular hydrogen bonding with hydroxyl group present in phenolic compounds and increase their solubility. This fact was proven by the study conducted by Zhou and Yu (2004). They found that 70% methanol extract showed the highest antioxidant activity. Likewise Pinelo et al. (2004) found that methanolic extraction in almond hull exhibited the strongest antioxidant activity and the order of antioxidant activity with different solvent was methanol > ethanol > water.

Ethanol extraction of earthworm paste of red worm and African night crawler showed the weakest antioxidant

activity. The inhibitory activity of African night crawler extract in 80% ethanol was 48.13%, while inhibitory activity of red worm extract in the same concentration was 47.37%. These results might be inconsistent with many studies indicating that ethanolic extraction was the effective solvents, and it gave a high yield. For example, Singh et al. (2002) found that the alcoholic extract of pomegranate peels exhibited the strongest antioxidant activity among all of the extracts. Mancini-Filho et al. (1998) also stated that an alcoholic extract showed the highest antioxidant ability than the aqueous solvents in cinnamon extracts. This contradiction may be due to many reasons. Firstly, differences in the DPPH radical scavenging activity of the methanolic and ethanolic extraction are produced from the differences in selectivity of solvents for extracting specific phenolic groups with various DPPH radical quenching activity (Zhao et al., 2006). Secondly, the extraction condition may affect generally the efficiency of antioxidant activity (Zhao and Yu, 2004). Finally, the extracts may include nonphenolic materials such as sugar, protein, pigments and organic acid which can interrupt throughout antioxidant assessment (Ronald et al., 2005).

The results revealed that African night crawler worm extract had the strongest inhibition activity than the red worm extract (Figure 2). The differences between antioxidant activity of African night crawler and red worm extract may be due to the fact that there is a relationship between chemical structure of phenolic compounds and their antioxidant activity. Many studies supported this fact. For examples, According to Kim and Lee (2004), radical scavenging activity of phenolic acids was promoted by additional hydroxy (OH) and methoxy group (OCH₃) groups on the aromatic ring of hydroxycinnamic acids. Similar results were reported by Baderschneider and Winterhalter (2001) who showed that cinnamic acid derivatives are possible antioxidants. Lopez-Velez et al. (2003) demonstrated the criteria in which the antioxidant quenching efficiency could be determined. The criteria included: orthodihydroxy structure, a 2,3 double bond and hydroxyl substitutions at positions three and five in the benzyl ring of cinnamic acid derivatives. Natella et al. (1999) also reported that OH-derivatives of cinnamic acids such as ferulic acids, caffeic acid and *p*-coumaric acid had more antioxidant activity than OH-derivatives of benzoic acids.

The comparison between total phenolic content (TPC) and antioxidant activity of the earthworm paste of the red worm and African night crawler indicate that there was equivocal correlation between TPC and antioxidant activity. Although, the TPC of red worm extract (237.73 mg/L) is higher than that of the African night crawler extract (214.87 mg/L), its corresponding antioxidant activity is lower. Moreover, the TPC of the red worm extract in 80% methanol (228.13 mg/L) and in 75% methanol was different, while their antioxidant activity was almost the same. In contrast, the TPC and antioxidant activity of the African night crawler extract was similar. These results

might be because the Folin-Ciocalteu method cannot quantify and determine all phenolic compounds in the samples (Naczka and Shahidi, 2004). Ronald et al. (2005) reported that many substances such as sugar, aromatic amines, sulfur dioxide and ascorbic acid may interfere with Folin-Ciocalteu assay leading to overestimation of total phenolics content. They also stated that many inorganic substances could react with Folin-Ciocalteu reagent leading to elevated phenolic concentration. Moreover, differential response of phenolic compounds in this assay relies on the number of phenolic groups they have. Commonly, polyphenol is more effective than monophenol, and methoxy groups can increase antioxidant activity of monophenol; however, for phenolic acids, addition of hydroxyl group can increase the antioxidant activity, leading to the differences in the antioxidant activity (Zhao et al., 2006).

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REFERENCES

- Alam N, Yoon KN, Lee KR, Shin PG, Cheong JC, Yoo YB (2010). Antioxidant activities and tyrosinase inhibitory effects of different extracts from *pleurotus ostreatus* fruiting bodies. *Mycobio.* 38: 295-301.
- Baderschneider B and Winterhalter P (2001). Isolation and characterization of novel benzoates, cinnamates, flavonoids, and lignans from Riesling wine and screening for antioxidant activity. *J. Agric. Food Chem.* 49: 2788-2798.
- Balamurugan M, Parthasarathi K, Cooper E, Ranganathan L (2007). Earthworm paste (*Lampito mauritii*, Kinberg) alters inflammatory, oxidative, haematological and serum biochemical indices of inflamed rat. *Eur. Rev. Medic. Pharmacol. Sci.* 11: 77.
- Balamurugan M, Parthasarathi K, Ranganathan LS, Cooper EL (2008). Hypothetical mode of action of earthworm extract with hepatoprotective and antioxidant properties. *J. Zhejiang Univ. Sci. B* 9:141-147.
- Cacace J and Mazza G (2003). Optimization of extraction of anthocyanins from black currants with aqueous ethanol. *J. Food Sci.* 68: 240-248.
- Castaneda-Ovando A, Pacheco-Hernández ML, Páez-Hernández ME, Rodríguez JA, Galán-Vidal CA (2009). Chemical studies of anthocyanins: A review. *Food Chem.* 113: 859-871.
- Cheng MB, Wang JC, Li YH, Liu XY, Zhang X, Chen DW (2008). Characterization of water-in-oil microemulsion for oral delivery of earthworm fibrinolytic enzyme. *J. Contr. Rel.* 129: 41-48.
- Dai J and Mumper RJ (2010). Plant phenolics: Extraction, analysis and their antioxidant and anticancer properties. *Mol.* 15: 7313-7352.
- Grdisa M, Popovic M, Hrzenjak T (2001). Glycolipoprotein extract (G-90) from earthworm *Eisenia foetida* exerts some antioxidative activity. *Comp. Biochem. Phys. Part A: Mol. Integ. Phys.* 128:821-825.
- Kapasakalidis PG, Rastall RA, Gordon MH (2006). Extraction of polyphenols from processed black currant (*Ribes nigrum* L.) residues. *J. Agric. Food Chem.* 54:4016-4021.
- Katalinic V, Možina SS, Skroza D, Generalić I, Abramović H, Miloš M (2010). Polyphenolic profile, antioxidant properties and antimicrobial activity of grape skin extracts of 14 *Vitis vinifera* varieties grown in Dalmatia (Croatia). *Food Chem.* 119: 715-723.
- Kim DO and Lee CY (2004). Comprehensive study on vitamin C equivalent antioxidant capacity (VCEAC) of various polyphenolics in scavenging a free radical and its structural relationship. *Crit. Rev. Food Sci. Nutr.* 44: 253-273.
- Li W, Wang C, Sun Z (2011). Vermipharmaceuticals and active proteins isolated from earthworms. *Pedobio.*, 54S: 54S-55S.
- Liu YQ, Sun ZJ, Wang C, Li SJ, and Liu YZ (2004). Purification of a novel antibacterial short peptide in earthworm *Eisenia foetida*. *Acta Biochimica et Biophysica Sinica*, 36: 297-302.
- Lopez-Velez, M, Martinez-Martinez F, Del Valle-Ribes C (2003). The study of phenolic compounds as natural antioxidants in wine. *Crit. Rev. Food Sci. Nutr.* 43: 33-244.
- Mancini-Filho J, Van-Koij A, Mancini D, Cozzolino F, Torres R (1998). Antioxidant activity of cinnamon (*Cinnamomum zeylanicum*, Breyne) extracts. *Bollettino Chimico Farmaceutico*, 137: 443.
- Naczka M and Shahidi F (2004). Extraction and analysis of phenolics in food. *J. Chromato. A*, 1054: 95-111.
- Nakajima N, Sugimoto M, Ishihara K (2003). Earthworm-serine protease: Characterization, molecular cloning, and application of the catalytic functions. *J. Mol. Catal. B: Enzy.* 23: 191-212.
- Natella F, Nardini M, Di Felice M, Scaccini C (1999). Benzoic and cinnamic acid derivatives as antioxidants: Structure-activity relation. *J. Agric. Food Chem.* 47, 1453-1459.
- Nepote V, Grosso NR, Guzman CA (2005). Optimization of extraction of phenolic antioxidants from peanut skins. *J. Sci. Food Agric.* 85: 33-38.
- Pinelo M, Rubilar M, Sineiro J, Nunez M (2004). Extraction of antioxidant phenolics from almond hulls (*Prunus amygdalus*) and pine sawdust (*Pinus pinaster*). *Food Chem.* 85:267-273.
- Prakash M, Balamurugan M, Parthasarathi K, Gunasekaran G, Cooper E, Ranganathan L (2007). Anti-ulceral and anti-oxidative properties of "earthworm paste" of *Lampito mauritii* (Kinberg). *Eur. Rev. Med. Pharmacol. Sci.* 11: 9-15.
- Ranganathan LS (2006). Vermibiotechnology: From soil health to human health. *Agro bios*, (India).
- Ronald L, Wu X, Schaich K (2005). Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* 53: 4290-4302.
- Singh R, Murthy KNC, Jayaprakasha G (2002). Studies on the antioxidant activity of pomegranate (*Punica granatum*) peel and seed extracts using in vitro models. *J. Agric. Food Chem.* 50:81-86.
- Spigno G, Tramelli L, De Faveri DM (2007). Effects of extraction time, temperature and solvent on concentration and antioxidant activity of grape Marc phenols. *J. Food En.* 81(1): 200-208.
- Turkmen S, Velioglu YS, Sari, F, Polat G (2007). Effect of extraction conditions on measured total polyphenol contents and antioxidant and antibacterial activities of black tea. *Mol.* 12: 484-496.
- Zhao J, Qi SP, Wu J, Li L, He RQ (2005). Earthworm fibrinolytic enzyme. *Stud. Natur.Prod. Chem.*, 30: 825-847.
- Zhao H, Dong J, Lu J, Chen J, Li Y, Shan L (2006). Effects of extraction solvent mixtures on antioxidant activity evaluation and their extraction capacity and selectivity for free phenolic compounds in barley (*Hordeum vulgare* L.). *J. Agric. Food Chem.* 54: 7277-7286.
- Zheng W and Wang SY (2001). Antioxidant activity and phenolic compounds in selected herbs. *J. Agric. Food Chem.* 49: 5165-5170.
- Zhou K and Yu L (2004). Effects of extraction solvent on wheat bran antioxidant activity estimation. *LWT-Food Sci. Technol.* 37: 717-721.