Optimization of Diplazium Esculentum Extract Using Pressurized Hot Water Extractor by Box-Behnken Design of Experiments and Its Antioxidative Behavior

A J Nur1, F K Khairul1,2*, M A Nuradibah1, S S S Noor3

1School of Bioprocess Engineering, Universiti Malaysia Perlis, 02600 Arau, Perlis, Malaysia.
2Centre of Excellence for Biomass Utilization, School of Bioprocess Engineering, Universiti Malaysia Perlis
3School of Chemical Sciences and Food Technology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor.

*Email: khairulfarihan@unimap.edu.my

Abstract. Pressurized hot water extraction (PHWE) was implemented in attempt to reduce the use of toxic organic solvent in extracting bioactive compounds from Diplazium esculentum. The antioxidant assays, which were the DPPH scavenging activity, total phenolic contents and total flavonoid contents were analyzed on PHW extracts. By applying Box-Behnken design under response surface methodology (RSM), the optimized condition for the best antioxidant activity of PHWE was at 175 °C, 21 min extraction time and 50 mL water volume added to 2 g of dried D. esculentum. The DPPH antiradical efficiency of D. esculentum give EC50 values of 1241.14 µg/mL and time to reach steady state (T EC50) was 79.83 min. Thus, the extract was categorized as slow in scavenging the DPPH free radicals.

1. Introduction
Diplazium esculentum (Retz.) Swartz also known as Athyrium esculentum (Retz.) Copel is in the family of Athyriaceae. This vegetable ferns (known as pucuk paku in Malaysia) grows wildly at moist or wet areas such as partially shaded river banks or homestead ground. The diploid plant occurs throughout Asia and Oceania (Indonesia, Philippines, Thailand, Papua New Guinea, Bangladesh, Japan and Fiji [1,2]. The fronds are use in traditional remedy to treat jaundice, constipation, earache, fever, measles and dermatitis [3,4].

D. esculentum were freshly used, boiled with water or soaked with organic solvents (hexane, acetone and alcohols) prior to analysis of the bioactive compounds in several studies [5,6,7]. However, there is no recent extraction method has been applied on D. esculentum up to this date. Thus, pressurized hot water extraction (PHWE) was applied in this recent study on D. esculentum due to the advantages of non-toxic, non-flammable and environmental friendly criteria of water as solvents.

PHWE refers to the extraction with water under pressure at the temperature between the boiling and critical temperature (100 and 374 °C respectively) [8]. The extraction of the bioactive compounds in plants could be affected by the plant species, extraction temperature, time, water-sample ratio and/or pressure4, [9, 10, 11].
This research focuses on the extraction of *D. esculentum* active compounds which contribute to DPPH antiradical activity. Optimization of the extraction condition of PHWE for the DPPH, total phenolic contents (TPC) and total flavonoid contents (TFC) will be analyzed using Box-Behnken design of experiment.

### 2. Materials and Methods

#### 2.1. Plant Material
*D. esculentum* were bought from local market in Arau, Perlis. The fronds and stems were dried overnight in the oven (Binder, Malaysia) at 60 °C and ground to a fine powder (< 65 µm) for the extraction processes.

#### 2.2. Optimization Using Design of Experiment
Optimization of extraction condition was done by using three-level three factor Box-Behnken design with total of 17 runs including five centre points under RSM. The analysis was carried out using Stat-Ease software (Design-Expert 7.1.5, 2008). The range of parameters (Table 1) used was based on the earlier screening results of the antioxidant assays at various PHWE condition.

#### Table 1: Range of parameters used for the optimization of antioxidant activity.

| Factors                        | Level  | Minimum limit | Maximum limit |
|--------------------------------|--------|---------------|---------------|
| Extraction temperature (°C)    | (-1)   | 125           | 175           |
| Extraction time (minutes)      | (+1)   | 10            | 30            |
| Volume of distilled water (mL) |        | 30            | 50            |

#### 2.3. DPPH Scavenging Activity
Antioxidant activity was determined using DPPH scavenging method [12].

#### 2.4. Total Phenolic Content (TPC)
The Folin-Ciocalteu colorimetric method with minor modification was used to determine TPC in *D. linearis* extracts [13].

#### 2.5. Total Flavonoid Content (TFC)
The total of flavonoids in *D. linearis* extracts were determined using aluminum chloride colorimetric method [13].

#### 2.6. DPPH Antiradical Efficiency
The DPPH antiradical efficiency of the optimized PHW extracts of *D. esculentum* and reference standard quercetin were determined using method reported by Sánchez-Moreno et al. [14].
2.7. Statistical Analysis
The results were presented as mean ± SEM of 6 independent experiments. Statistical values (P<0.05) were determined by Student’s t-test using GraphPad Prism 5.02, GraphPad Software Inc.

3. Results and Discussion

3.1. Optimization of Three Responses Using Box-Behnken Design

3.1.1. Effects of Parameters on DPPH Scavenging Activity

The parameters units in the design model (Table 2) were coded as A, B and C for extraction temperature, extraction time and volume of distilled water respectively. Analysis of variance shows the reduced two factors interaction design model was significant for DPPH scavenging activity response (p < 0.001) with no lack of fit. The Adj-R² value for the respond was 0.8089 much higher than the Pred-R² (0.4609) could be due to large block effects.

The Adj-R² is a statistic that is adjusted for the size or number of factors in the experiment and can decrease if insignificant variables are added to the design model. Reducing insignificant term in the model would produce a more precise data predictor [15]. The interaction between A and B was insignificant, therefore was eliminated from the design model.

The main effect of scavenging activity was extraction temperature (A) (P < 0.001). While extraction time (B) and water volume (C) on its own has no significant effects on the DPPH test but they were significantly involved in the interaction of AC and BC. Thus, terms B and C were not eliminated from the model design.

The equation of the reduced model term was:
DPPH = 70.18235 + 3.23750A + 0.38750B - 0.90000C + 1.75000AC + 2.10000BC

Referring to the interaction of A and C at three level of B (-1, 0 and +1) on DPPH radical scavenging activity, not obvious increment on DPPH response at low level of C (-1.00 C) from low to high level of A, but significant increment can be seen at high level of C (+1.00 C). Both level of water volume (C), require high temperature for good DPPH assay result.

In AC interaction, at high level of B (+1.00 B), the highest DPPH test response was at high level of C with prediction of 76.76% scavenging activity (Figure 1c). Lowering B level to 0.00 B (Figure 1b), DPPH test response was increased at low level of C, but still the highest point was at +1.00 C with reduced DPPH test prediction to that of 74.27%. At lowest level of B (-1.00 B) (Figure 1a), the DPPH response was highest at -1.00 C with 74.28% and while +1.00 C showed further reducing DPPH response. Thus, good DPPH scavenger compounds can best be extracted at high temperature, and the extraction time was highly influenced by water volume. This is supported by the DPPH antioxidant activity of kaffir lime fruit peel. The DPPH scavenging activity was increasing with extraction temperature up to 200 °C but no significant effect of extraction time on PHW extraction condition [16].
Table 2: The Box-Behnken design model with three responses

| Std | Run | A: Temperature (°C) | B: Time (min) | C: Water volume (mL) | Response 1 | Response 2 | Response 3 |
|-----|-----|---------------------|---------------|-----------------------|------------|------------|------------|
| 4   | 1   | 1                   | 1             | 0                     | 73.9       | 79.6       | 14.5       |
| 9   | 2   | 0                   | -1            | -1                    | 71.1       | 80.1       | 14.5       |
| 5   | 3   | -1                  | 0             | -1                    | 70.7       | 90         | 13.2       |
| 14  | 4   | 0                   | 0             | 0                     | 70.1       | 85.3       | 14.6       |
| 6   | 5   | 1                   | 0             | -1                    | 74.5       | 86.7       | 13.3       |
| 2   | 6   | 1                   | -1            | 0                     | 72.7       | 78.3       | 14.7       |
| 10  | 7   | 0                   | 1             | -1                    | 68.2       | 85.4       | 16.6       |
| 17  | 8   | 0                   | 0             | 0                     | 69.6       | 84.5       | 14.9       |
| 16  | 9   | 0                   | 0             | 0                     | 70.9       | 85.1       | 14.1       |
| 15  | 10  | 0                   | 0             | 0                     | 70.1       | 85.3       | 14.6       |
| 8   | 11  | 1                   | 0             | 1                     | 73.8       | 94.5       | 16.1       |
| 1   | 12  | -1                  | -1            | 0                     | 68         | 79.6       | 10.9       |
| 7   | 13  | -1                  | 0             | 1                     | 63         | 78.5       | 10.3       |
| 11  | 14  | 0                   | -1            | 1                     | 67.5       | 88.8       | 14.8       |
| 12  | 15  | 0                   | 1             | 1                     | 73         | 96.8       | 14.4       |
| 3   | 16  | -1                  | 1             | 0                     | 67.3       | 79.7       | 13.9       |
| 13  | 17  | 0                   | 0             | 0                     | 68.7       | 83.9       | 14.2       |

\(^a\) scavenging activity (%)  
\(^b\) µg catechin equivalent/mg dry extracts  
\(^c\) µg rutin equivalent/mg dry extracts

Figure 1: The interaction of extraction temperature and volume of water effects on DPPH scavenging activity response at: a; 10 min, b; 20 min, c; 30 min. The terms -1.000 and +1.000 codes for low and high level of: A; temperature (125 °C and 175 °C), B; extraction time (10 and 30 min), C; water volume (30 and 50mL).
3.1.2. Effects of Parameters on TPC

Quadratic model was suggested by Design Expert for the TPC response. Analysis of variance shows the model was significant to the response with significant lack of fit. Statistically, the three factors (A, B and C) seems to be insignificant model terms (P > 0.05), therefore reducing the factors resulting a significant model design with $R^2$ value of 0.6342. Low value of $R^2$ does not mean the regression model cannot be acceptable. This is because, the $R^2$ value will keep increasing by the addition of another model terms including insignificant terms which would results in poor prediction on new data\cite{2}. In TPC response, AC and $C^2$ are significant model terms with the following equation:

$$TPC = 82.367 + 1.413A + 2.05C + 4.825AC + 5.233C^2$$

The interaction of factor B with the factor A and C respectively shows no significant effects on model design, thus B was eliminated from the model term. However, A and C were involved in an interaction (Figure 2a). At low level of C (-1.000 C), TPC value will decrease with increasing of A whereas, at high level of C (+1.000 C), TPC value will increase along with A.

From Figure 2b, TPC will increase with elevated temperature but no effects on extraction time variation. This finding was supported by the result of parallel increment of TPC and extraction temperature in bitter melon subcritical water extraction. The suitable temperature was in the range between 150-200 °C. Higher extraction temperature may cause degradation of phenolic compounds\cite{17}.

3.1.3. Effects of Parameters on TFC

Analysis of variance on quadratic model applied for TFC response shows the parameters were significant (P < 0.001) for the model design with no lack of fit except for water volume (C). However, C was not eliminated in the model design because there were interaction between A (temperature) and B (time) respectively with significant model terms with the following equation:

$$TFC = 14.48 + 1.29A + 0.56B - 0.25C - 0.80AB + 1.42AC - 0.63BC - 1.41A^2 + 0.44B^2 + 0.16C^2$$
In BC interaction at low extraction temperature (-1.000 A) (Figure 3a), more time is required (+1.000 B) for the lowest water volume (-1.000 C) to get high TFC with prediction of 15.96 µg rutin equivalent/mg dry extract. By increasing the temperature to 0.000 A, the TFC was increasing at both level of C factors (Figure 3b). The highest prediction was 16.43 µg rutin equivalent/mg dry extract at high level of both B and C. The results was further increased at higher temperature (+1.000 A) for high level of C with design point of 16.91 µg rutin equivalent/mg dry extract (Figure 3c). While at low level of C, the TFC was reducing. Thus, temperature highly influenced TFC result with interaction on extraction time and water volume.

Figure 3: The interaction of extraction time and volume of water effects on TFC at: a; 125 °C, b; 150 °C, c; 175 °C. The terms -1.000 and +1.000 codes for low and high level of: A; temperature (125 °C and 175 °C), B; extraction time (10 and 30 min), C; water volume (30 and 50 mL).

3.1.4. Validation of the Optimized Condition

Suggested condition for optimum antioxidant activity of D. esculentum PHW extraction was 175 °C extraction temperature at 21 min and 50 mL of distilled water with desirability of 0.828. Conformation run was done on optimized extraction condition. The three analyses were done on the optimized extract and it was shown that DPPH scavenging activity of actual results was lower than predicted (67.65 ± 1.2 and 74.5% respectively). The TPC of real experiment is much higher than predicted (125.7 ± 16.4 µg and 95.89 µg catechin equivalent/mg dry weight respectively) while TFC
of actual result was slightly lower than the predicted (11.12 ± 0.58 µg and 15.53 µg rutin equivalent/mg dry weight respectively). The design model of TPC has low pred-R², which could explain the cause of huge differences between the predicted and actual result. It can be concluded that, longer extraction time gave better results for DPPH scavenging activity and total phenolic compounds but less time needed for high total flavonoid contents. Increasing the extraction time could increase the contacts time between solvents and samples.

3.2. DPPH Antiradical Efficiency

The DPPH scavenging activity of standard quercetin at four different concentrations (i.e. 5, 10, 20 and 50 µg/mL) from 1 to 120 min were observed (Figure 4A). The scavenging activities were increasing with standard concentration. At low concentrations, the scavenging activities were less than 30% activity, while at higher concentration reaching almost 80% activities. At low concentration, the time needed to reach the steady states was longer (120 min) and at 20 µg/mL, took lesser time (80 min). At 50 µg/mL, only 9 min was required to reach the steady state. Similar trends were seen in D. esculentum extracts at various and higher concentrations than pure quercetin (500 – 2500 µg/mL) (Figure 4B). At 1000 µg/mL and lower concentrations, the DPPH scavenging activities were lower than 55% activities. The highest activities were at the highest concentration (2500 µg/mL) with 77% activities. At low concentrations, the time required to reach steady states were longer (100-120 min), while at 1500, 2000, and 2500 µg/mL needed 80, 50 and 20 min respectively to reach steady states.

Thus, the kinetic behaviour of antioxidant depends on the concentration or the amount of the compounds to react with free radicals. Rapid kinetic behaviour occur when the percentage of remaining DPPH reaching a steady state in less than 5 min as of ascorbic acid. Intermediate kinetic behaviour occur in within 5 to 30 min, while longer time (> 30 min) was categorized as slow behaviour [18]. From the results, it can be concluded that the kinetic behaviour of quercetin and D. esculentum were intermediate at high concentration and slow at low concentration. The kinetic behaviours of quercetin and D. esculentum extracts follow a general multiplicative model:

\[
\ln \left[ \% \text{ DPPH remaining} \right] = b \ln \text{ time} + \ln \ a
\]

where \( b \) is the slope and \( a \) is the intercept. The slope determined the rate of percentage of remaining DPPH (% DPPH remaining/min) after scavenged by the antiradicals (quercetin and D. esculentum extracts).

The slope is steeper at higher concentration and the remaining DPPH radicals are lower [14]. From the results in Table 3, the slope is steeper with increasing sample concentrations, which is the
rate of scavenging antiradical is higher. However, at 50 µg/mL (quercetin) and 2000 and 2500 µg/mL \((D. \text{ esculentum})\) the slope is less steep because at these concentrations, the antiradical activities have reached plateau at early time (9, 50 and 20 min respectively). The remaining DPPH radicals were reducing at higher concentration at the time to reach steady state because there are more antiradicals compounds available to scavenge the DPPH radicals at a time.

Table 3: Kinetic behaviour of quercetin and \(D. \text{ esculentum}\)

| Sample          | Concentration (µg/mL) | Slope (% DPPH/min) | Correlation coefficients | Remaining % DPPH at steady state |
|-----------------|-----------------------|--------------------|--------------------------|---------------------------------|
| Quercetin       | 5                     | -0.07              | -0.991                   | 77.34                           |
|                 | 10                    | -0.102             | -0.995                   | 64.99                           |
|                 | 20                    | -0.298             | -0.983                   | 23.75                           |
|                 | 50                    | -0.02              | -0.892                   | 23.82                           |
| \(D. \text{ esculentum}\) | 100                | -0.067             | -0.967                   | 79.87                           |
|                 | 500                   | -0.095             | -0.986                   | 68.01                           |
|                 | 1000                  | -0.178             | -0.994                   | 45.74                           |
|                 | 1500                  | -0.275             | -0.997                   | 28.76                           |
|                 | 2000                  | -0.123             | -0.782                   | 26.92                           |
|                 | 2500                  | -0.014             | -0.996                   | 23.26                           |

\(^a\) Value mean is ± standard deviation.
\(^b\) Time needed to reach the steady state to EC\(_{50}\).

The amount of antioxidant required to decrease the initial DPPH radical concentration by 50% is known as EC\(_{50}\). Since the DPPH antiradical activity is dose dependent process, the antiradical efficiency was determined by EC\(_{50}\) values of each sample (Table 4). The EC\(_{50}\) value was determined by plotting the concentrations of antioxidants against the percentages of remaining DPPH radicals [19]. The lower the EC\(_{50}\) value, the higher the antioxidant power of the compound.

Phenolic compounds have diverse EC\(_{50}\) values depends on the structure and the mechanism involves to scavenge the DPPH radicals [20,21]. The efficiency of antiradicals are not solely dependent on the EC\(_{50}\) of the compounds because the rate of reaction depends on the concentration. Thus, TEC\(_{50}\) were determined (Table 4) which is the time for the sample at EC\(_{50}\) to reach equilibrium [22].

Table 4: The concentration needed to reduce DPPH concentration by 50% and their kinetic classification.

| Sample          | EC\(_{50}\)^a (µg/mL) | Ranges of times at the steady state (min) for the sample concentrations | TEC\(_{50}\)^a,b (min) | Classification |
|-----------------|------------------------|-----------------------------------------------------------------------|------------------------|----------------|
| Quercetin       | 15.98 ± 9              | 9.0-120.0                                                             | 95.29 ± 0.1            | Slow           |
| \(D. \text{ esculentum}\) | 1241.14 ± 7           | 20.0-120.0                                                            | 79.83 ± 0.5            | Slow           |

\(^a\) Value mean is ± standard deviation.
\(^b\) Time needed to reach the steady state to EC\(_{50}\).
The lower the EC$_{50}$, the lower the TEC$_{50}$ but in this study, TEC$_{50}$ value for both samples were almost similar although the EC$_{50}$ value were significantly different. Similar condition had been observed in gallic acid and (-)-epicatechin, where EC$_{50}$ for both compound were quite similar but the TEC$_{50}$ value for gallic acid was far lower than (-)-epicatechin$^{14}$. This shows that there was uncertainty in the determination of the antioxidant activity using EC$_{50}$ and TEC$_{50}$ values. Therefore, the efficiency of phenolic compounds to scavenge the DPPH were further determined by antiradical efficiency equation:

\[
\text{Antiradical efficiency} = \frac{1}{\text{EC}_{50} \cdot \text{TEC}_{50}}
\]

The antiradical efficiency of quercetin (0.65x10$^{-3}$) was higher compared to D. esculentum (0.01x10$^{-3}$) and both were classified as low antiradical efficiency (Table 5). The percentage of remaining DPPH radicals against the concentration of these two samples were expressed by exponential model.

\[
\ln [\% \text{DPPH remaining}] = b \ [\text{antioxidant}] + \ln a
\]

From this model, the slope of linear regression of quercetin was higher than D. esculentum extracts. The steeper the slope, the lower EC$_{50}$ would give higher antiradical efficiency [14]. Therefore, the antiradical efficiency of D. esculentum was lower than quercetin.

| Sample       | Slope (x 10^{-3}) | Correlation coefficients | Antiradical efficiency (x 10^{-3}$^a$) | Classification |
|--------------|-------------------|--------------------------|----------------------------------------|----------------|
| Quercetin    | -0.083            | -0.960                   | 0.65                                   | Low            |
| D. esculentum| -0.001            | -0.958                   | 0.01                                   | Low            |

$^a$ Antiradical efficiency = 1/EC$_{50}$×TEC$_{50}$

**Acknowledgements**

The author would like to acknowledge the support from the Fundamental Research Grant Scheme (FRGS) under a grant number of FRGS/1/2016/TK02/UNIMAP/03/2 from the Ministry of Higher Education Malaysia.

**References**

[1] Noweg, T.; Abdullah, A.R.; Nidang, D. Forest plants as vegetables for communities bordering the Crocker Range National Park. *ASEAN Review of Biodiversity and Environmental Conservation (ARBEC)*. 2003, 18.

[2] Sarker, S.K.; Hossain, A.B.M. Pteridophytes of greater Mymensingh district of Bangladesh used as vegetables and medicines. *Bangladesh Journal of Plant Taxonomy*. 2009, 16(1), 47-56.

[3] Baas, P.; Kalkman, K.; Gessink, R. *The plant diversity of Malesia*. Springer Netherlands. 1990, 1, 420

[4] Bernama, *Promoting local herbal industry to local market*, in *Borneo Post*. 2009, Malaysia. p. B13.

[5] Roy, S.; Hazra, B.; Mandal, N.; Chaudhuri, T.P. Assessment of the Antioxidant and Free Radical Scavenging Activities of Methanolic Extract of Diplazium esculentum. *Int. J. Food Prod. 2013*, 16(6), 1351-1370.

[6] Tongco, J.V.; Villaber, R.A.; Aguda, R., Razal, Ramon. Nutritional and Phytochemical Screening, and Total Phenolic and Flavonoid Content of Diplazium esculentum (Retz.) Sw. from the Philippines. *J Chem Pharm Res*. 2014, 6(8):238-242
[7] Akter, S.; Hossain, M.M.; Ara, I.; Akhtar, P. Investigation of in vitro antioxidant, antimicrobial and cytotoxic activity of Diplazium esculentum (Retz.) Sw. Int. J. Adv. Pharm. Biol. Chem. 2014, 3(3), 723-733.

[8] Teo, C. C.; Tan, S. N.; Yong, J. W. H.; Hew, C. S.; Ong, E. S. Pressurized hot water extraction (PHWE). J. Chromatogr. A. 2010, 2484-2494.

[9] Rangsriwong, P.; Rangkadilok, N.; Satayavivad, J.; Goto, M.; Shotipruk, A. Subcritical water extraction of polyphenolic compounds from Terminalia chebula Retz. fruits. Sep. Purif. Technol. 2009, 66, 51-56.

[10] Gbashi, S.; Njobeh, P.; Steenkamp, P.; Tutu, H.; Madala, N. The effect of temperature and methanol-water mixture on pressurized hot water extraction (PHWE) of anti-HIV analogues from Bidens pilosa. Chem Cent J. 2016, 10(1), 1752-1789.

[11] Baharuddin, N. A. F.; Nordin, M. F. M.; Morad, N. A.; Rasidek, N. A. Pressurized hot water extraction of phenolic and antioxidant activity of Clinacanthus nutans leaves using accelerated solvent extractor. Aust. J. Basic & Appl. Sci. 2017, 11(3), 56-63.

[12] Rajesh, K.D.; Vasantha, S.; Panneerselvam, A.; Valsala Rajesh, N.; Jeyathilakan, N. Phytochemical Analysis, in vitro Antioxidant Potential and Gas Chromatography Mass Spectrometry Studies of Dicranopteris Linearis. Asian J. Pharm. Clin. Res. 2016, 9, 220.

[13] Vijayalaxmi, S.; Jayalakshmi, S. K.; Sreeramulu, K. Polyphenols from different agricultural residues: extraction identification and their antioxidant properties. J. Food Sci. Technol. 2015, 52(5), 2761-2769.

[14] Sánchez-Moreno, C.; Larrauri, J.A.; Saura-Calixto, F. A procedure to measure the antiradical efficiency of polyphenols. J. Sci. Food Agric. 1998. 76(2): p. 270-276.

[15] Montgomery, D.C. Design and analysis of experiments. 5th ed. 1997, United States of America: John Wiley & Sons, Inc. 699.

[16] Khuwijitjaru, P.; Chaloooddong, K.; Adachi, S. Phenolic content and radical scavenging capacity of kaffir lime fruit peel extracts obtained by pressurized hot water extraction. Food Sci. Technol. Res. 2008. 14(1): p. 1-4.

[17] Budrat, P.; Shotipruk, A. Enhanced recovery of phenolic compounds from bitter melon (Momordica charantia) by subcritical water extraction. Sep. Purif. Technol. 2009, 66(1), 125-129.

[18] Brand-Williams, W.; Cuvelier, M.E.; Berset, C. Use of a free radical method to evaluate antioxidant activity. LWT-Food Sci Technol. 1995. 28(1): p. 25-30.

[19] Villaño, D.; Fernández-Pachón, M. S.; Moyá, M. L.; Troncoso, A. M.; García-Parrilla, M.C. Radical scavenging ability of polyphenolic compounds towards DPPH free radical. Talanta, 2007. 71(1), 230-235.

[20] Bondet, V.; Brand-Williams, W.; Berset, C. Kinetics and Mechanisms of Antioxidant Activity using the DPPH Free Radical Method. Lebensmittel-Wissenschaft und-Technologie. 1997, 30(6), 609-615.

[21] Tsimogiannis, D.I.; Oreopoulou, V. The contribution of flavonoid C-ring on the DPPH free radical scavenging efficiency. A kinetic approach for the 3’,4’-hydroxy substituted members. Innov. Food Sci. Emerg. Technol. 2006, 7(1-2), 140-146.

[22] Tsao, R.; Deng, Z. Separation procedures for naturally occurring antioxidant phytochemicals. J. Chromatogr. B. 2004, 812(1-2), 85-99.