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The Factors Affecting the Extraction Condition for Neuroprotective Activity of *Centella asiatica* Evaluated by Metal Chelating Activity Assay

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Abstract: The study was aimed to evaluate the metal chelating activity of CA which will first be assessed for neuroprotective property. Further, response surface methodology will be used to optimize the extraction parameters to yield an optimum metal chelating activity of CA by minimizing the cost of extraction. *Centella asiatica* (CA) is a rich antioxidant candidate was studied for its potential as a neuroprotective agent to fight against oxidative damage caused by Reactive Oxygen Species (ROS) towards neuronal cells which eventually lead to neurodegenerative diseases such as Alzheimer's and Parkinson disease. The limited number of neuroprotective study carried out so far indicated that metal (iron) chelation therapy could be a viable neuroprotective approach for neurodegenerative disorders. Methanolic and aqueous extract of CA was obtained by conventional soxhlet extraction (temperature: 40-60°C, ratio: 1:30 (w/v), time: 24 h). The IC_{50} of methanolic and aqueous extract obtained was 0.26 and 0.69 mg mL^{-1}, respectively. Further, Response surface methodology was used to determine the optimum CA extraction condition that gives optimum metal chelating activity. Experiments were designed according to central composite design with four factors (time, temperature, ratio of raw material to solvent and agitation speed) and six central points that derived total 30 runs. In conclusion, run 17 (temperature: 25°C, ratio: 1:45 (w/v), time: 1.5 h and speed: 200 rpm) gave the optimum chelating activity with IC_{50} 0.093 mg mL^{-1}.

Key words: *Centella asiatica* (CA), response surface methodology, metal chelating activity

INTRODUCTION

Locally known as pegaga, *Centella asiatica* (CA) had an enormous health benefit such as wound healing agent and brain stimulant (Kathy, 2000), bronchitis, asthma, dysentery, leucorrhoea, kidney trouble, urethritis, antiallergic and anticancer purposes, curing leucorrhea and toxic fever (Zainol *et al*., 2003), ability to improve venous insufficiency (Shinomol and Muralidhara, 2008). Its habitat distributed in sunny, moist grounds in all parts of the world (South East Asia, Australia, Africa, Brazil, Europe etc.) (Satake *et al*., 2007).

Neurodegenerative diseases had been reported to affect though a small world population its increase in diagnosed patient is alarming. The twentieth century witnessed a significant demographic change in the human population of the industrialized world that is currently followed by a similar shift of life expectancy to upper age ranges in Asia, Africa and Middle and South America (Beal *et al*., 2005). It was reported in local newspaper Utusan Malaysia about 35 million people around the world will be affected with AD or other dementia on 2010 and only a limited number will attain any treatment (REUTERS, 2009).

The main pathologies of neurodiseases such as Parkinson's, Alzheimer's, Huntington, multiple sclerosis and etc. is the buildup of iron at sites where the neurons deteriorate (LeVine *et al*., 2004). The probable important relevance in clinical therapeutics of progressive neurodegenerative diseases is the chelation of free cellular ferric and ferrous ions which are required for the generation of reactive oxygen radicals by the Fenton and Haber-Weiss reactions (Mandel and Youdim, 2004). The limited number of neuroprotective studies carried out so far indicates that iron chelation therapy could be a viable neuroprotective approach for neurodegenerative disorders (Mandel *et al*., 2006).

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Response Surface Methodology (RSM) is a statistical procedure frequently used for optimization studies. It uses quantitative data from an appropriate experimental design to determine and simultaneously solve multivariate problems. Equations describe the effect of test variables on responses, determine interrelationships among test variables and represent the combined effect of all test variables in any response. This approach enables an experimenter to make efficient exploration of a process or system (Madamba, 2002).

MATERIALS AND METHODS

Materials: The CA was purchased from Pasar Borong Selayang. An analytical grade solvents methanol was purchased from Fisher Scientific and ethanol from J. Kollin Corporation, Germany. The chemical reagent iron (II) sulfate heptahydrate ($FeSO_4$), 4,4'-[3-(2-pyridinyl)-1,2,4-triazine-5,6-diyl]bis (ferrozine) and Clioquinol was purchased from Aldrich, USA. Neuroblastoma cell line, SH-SY5Y was purchased from ATCC, USA.

This study was conducted at International Islamic University Malaysia, Kulliyyah Engineering, Department of Biotechnology Engineering and Forest Research Institute Malaysia, Drug Discovery Center from 2007 to 2009.

Preparation of CA: The CA was dried in drying oven at 50°C until the moisture content of the plants reached below 10%. Further it was ground in an electric grinder (Kinematics, Swiss) to obtain a fine powder. The powder form of CA was stored in 4°C until further use.

Soxhlet extraction: The CA was subjected to extraction in which 500 g of CA wrapped with muslin-cloth and added to thimble-holder and a total of 4 L of solvent (methanol or water) was added to solvent flask and to the thimble-holder holding the plants. The extraction was carried out for 24 h. The solvent was removed from extract by evaporation process using rotary evaporator at 40°C and at a reduced pressure.

Assay of metal chelating activity: The extract dissolved in ethanol (500 μL) was added to 2.5 mM $FeSO_4$ (250 μL) and vortex briefly for 10 sec. The mixture was then added with 6 mM ferrozine (250 μL) vortex briefly again for 10 sec and allowed to equilibrate for 10 min in room temperature. The absorbance of the mixture (formation of the ferrous iron-ferrozine complex) was measured at 562 nm (Dinis *et al*., 1994). The ability of the Clioquinol and CA extract to chelate ferrous ion was calculated relative to the control (consisting of iron and ferrozine only) using the Eq. 1 (Wu *et al*., 2007):

$$\text{Chelating effect (\%)} = \frac{(\text{Absorbance of control} - \text{Absorbance of sample})}{\text{Absorbance of control}} \times 100 \quad (1)$$

Design of Experiment (DoE): Design of Experiments (DoE) was able to optimize the extraction parameters of CA to obtain the optimum metal chelating activity. Central composite design was used to process the optimization procedure following analysis with Response Surface Methodology (RSM). Four independent variables had been chosen to be involved in the extraction process which is ratio of raw material to solvent, speed of agitation, time and temperature to assist the extraction process (Table 1). Face-centered composite had designed 30 experimental runs including 6 replicates at the center point to evaluate the combined effect of the independent variables (Table 2).

Table 1: Experiment independent variables

		Level and range		
Variables	Factor code	-1	0	1
Temperature (h)	A	25	30	45
Speed (rpm)	B	100	150	200
Ratio (g:mL)	C	1:35	1:40	1:45
Time (h)	D	0.5	1.0	1.5

Table 2: Face centered, central composite design setting with the independent variables and their responses in CA

Run No.	A: Temp (°C)	B: Speed (rpm)	C: Ratio (g:mL)	D: Time (h)	$1/IC_{50}$	IC_{50} (mg mL^{-1})
1	30	150	40	1	1.72	0.5814
2	30	150	40	1	1.429	0.6998
3	35	100	35	0.50	5.2	0.1923
4	30	150	40	1	1.55	0.6452
5	30	150	40	1.5	1.96	0.5102
6	35	200	35	0.50	2.4	0.4167
7	30	150	40	1	1.3426	0.7448
8	30	150	35	1	0.84	1.1905
9	30	150	40	0.5	1.57	0.6369
10	25	100	45	0.50	2.3	0.4348
11	35	200	45	1.50	2.703	0.3700
12	35	100	45	0.50	2.01	0.4975
13	25	100	35	0.50	4.6	0.2174
14	35	150	40	1	4.56	0.2193
15	30	200	40	1	1.399	0.7148
16	30	150	45	1	0.94	1.0638
17	25	200	45	1.50	10.753	0.0930
18	35	200	35	1.50	1.98	0.5051
19	30	150	40	1	1.49	0.6711
20	35	100	35	1.50	0.84	1.1905
21	30	150	40	1	1.49	0.6711
22	25	200	45	0.50	3	0.3333
23	35	200	45	0.50	0.29	3.4483
24	25	150	40	1	7.98	0.1253
25	25	100	45	1.50	3.49	0.2865
26	25	100	35	1.50	1.12	0.8929
27	25	200	35	0.50	4.167	0.2400
28	30	100	45	1.50	0.098	10.2041
29	35	100	45	1.50	0.93	1.0753
30	25	200	35	1.50	4.35	0.2299

RESULTS AND DISCUSSION

Metal chelating activity was performed on CA which had been extracted using soxhlet method. Methanolic extract showed favorable activity with IC_{50} = 0.26 mg mL^{-1} meanwhile IC_{50} = 0.69 mg mL^{-1} for aqueous extract (Fig. 1). As the methanolic extract showed approving activity it was brought forward for an optimization procedure. Design-Expert version 6.0.8 was used in evaluation of the extraction optimization process. Mathematical models were evaluated for each response by means of multiple regression analysis. The model statistical significant was checked by F-test and the analysis of variance (ANOVA) (Table 3) by utilizing the quadratic model as suggested by the software. The F-value of 23.80 and the p-value of <0.0001 demonstrated the significant of the regression with 99% confidence level. There is only a 0.01% chance that a Model F-Value this large could occur due to noise. The values of Prob>F less than 0.0500 indicate model terms are significant (Kabbashi *et al*., 2009).

Table 3: ANOVA for the response surface quadratic polynomial model

Source	Sum of squares	df	Mean square	F-value	Prob>F
Model	146.49	14	10.46	23.80	<0.0001*
A	24.14	1	24.14	54.92	<0.0001
B	6.07	1	6.07	13.81	0.0021
C	0.047	1	0.047	0.11	0.7484
D	0.37	1	0.37	0.85	0.3719
A^2	45.63	1	45.63	103.80	<0.0001
B^2	4.55	1	4.55	10.34	0.0058
C^2	3.63	1	3.63	8.25	0.0116
D^2	0.25	1	0.25	0.56	0.4658
AB	9.56	1	9.56	21.74	0.0003
AC	5.99	1	5.99	13.63	0.0022
AD	5.17	1	5.17	11.76	0.0037
BC	2.96	1	2.96	6.73	0.0203
BD	19.49	1	19.49	44.33	<0.0001
CD	21.05	1	21.05	47.89	<0.0001
Residual	6.59	15	0.44		
Lack of fit	6.51	10	0.65	40.29	0.0004*
Pure error	0.081	5	0.016		
Cor total	153.08	29			

*Significant

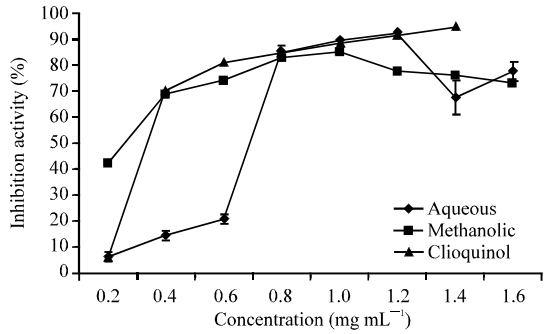

Fig. 1: Graph representing metal chelating activity of Clioquinol, methanolic and aqueous extract of CA

The response function (y) measured the $1/IC_{50}$ value of the metal chelating activity of CA. These value was related to the variables (A, B, C, D) by a second degree polynomial using the Eq. 2.

Final equation in terms of coded factors: IC_{50} = +1.79-1.16*A + 0.58*B +0.051*C +0.14*D +4.20*A^2 – 1.32*B^2-1.18*C^2-0.31*D^2-1.32*B^2-1.18*C^2-0.31*D^2-0.77*A*B – 0.61*A*C – 0.57*A*D +0.43*B*C + 1.10*B*D + 1.15*C*D (2)

The coefficients of the polynomial were represented by a constant term, A, B, C and D (linear effects), A^2, B^2, C^2 and D^2 (quadratic effects) and AB, AC, AD, BC, BD and CD (interaction effects). The ANOVA outcome in Table 3 were generated and the effect and regression coefficients of individual linear, quadratic and interaction terms were determined. The significances of all terms in the polynomial were judged statistically by computing the F-value at a probability (p) of 0.001, 0.01 or 0.05. In this case A, B, A^2, B^2, C^2, AB, AC, AD, BC, BD, CD are significant model terms. On the other hand, values greater than 0.1000 indicate the model terms are not significant (Kabbashi *et al*., 2009). The regression coefficients were then used to make statistical calculation to generate contour maps from the regression models. R^2 is a measure of the amount of variation around the mean explained by the model and equal to 0.9569.

Figure 2 shows the interaction between temperature and speed while holding constant ratio of raw material to solvent and time at 1g to 40 mL and 1 h, respectively. The increased in temperature decreased the

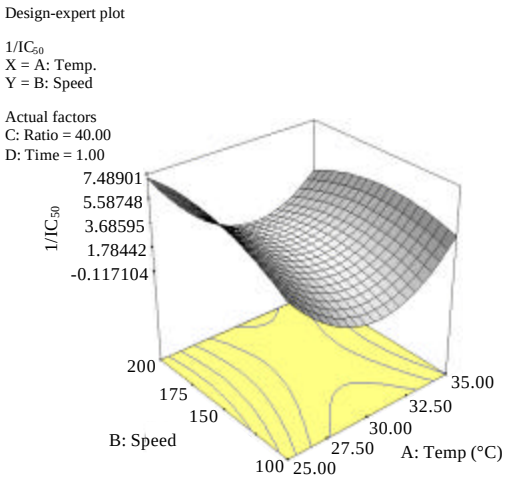

Fig. 2: Three-dimensional plot of the response surface for chelating activity in ratio of raw material to solvent at 1 g: 40 mL and time for extraction for 1 h

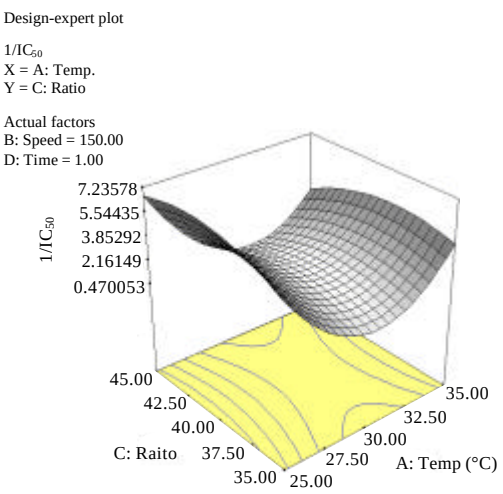

Fig. 3: Three-dimensional plot of the response surface for chelating activity with speed of extraction process at 150 rpm and time for extraction for 1 h

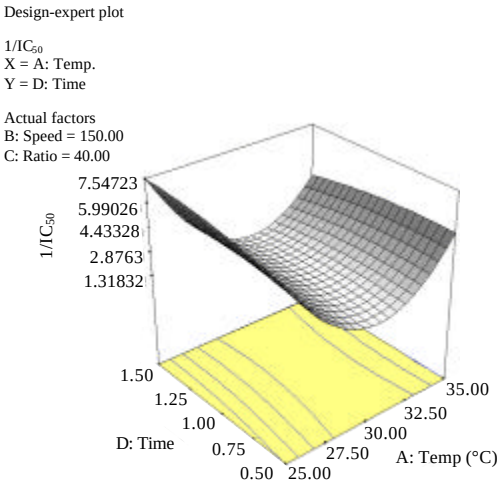

Fig. 4: Three-dimensional plot of the response surface for chelating activity with speed of extraction process at 150 rpm and ratio of raw material to solvent at 1 g: 40 mL

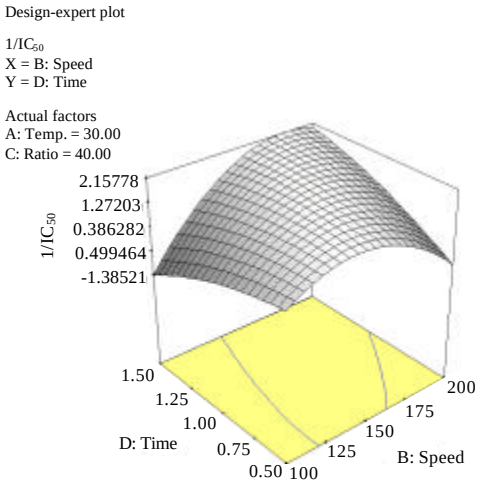

Fig. 5: Three-dimensional plot of the response surface for chelating activity with temperature during the extraction process at 30°C and the ration of raw material to solvent at 1 g: 40 mL

$1/IC_{50}$ value. At temperature of 30°C and above, however an increased in the activity of chelating was observed. In the other hand, a continuous increased in $1/IC_{50}$ value was observed with agitation during the extraction process of CA from 100 to 200 rpm.

Similar pattern of interaction also observed in Fig. 3 with increased in temperature reduced the chelating activity up to temperature of 30°C and thereafter increased the activity up to temperature of 35°C. The ratio of raw material to solvent of 35 mL to 45 mL continuously increased the chelating activity.

The increased in time did not affect the chelating activity while an increased and decreased in chelating activity was observed in temperature range of 25 to 35°C. Time did not play a significant role in increasing the metal chelating activity with respect to interaction with temperature (Fig. 4).

The interaction between time and speed at constant temperature of 30°C and ratio of 1 g to 40 mL gave the following plot as in Fig. 5. The regression analysis showed negative effect of chelating activity at time of 0.50 to 1.50 h. The speed influenced the chelating activity by increasing at 100 rpm and decreasing 150 to 200 rpm.

Many factors such as solvent composition, extraction time, extraction temperature (Wettasinghe and Shahidi, 1999), solvent to solid ratio (Cacace and Mazza, 2003) and extraction pressure (Cacace and Mazza, 2002), among others, may significantly influence the extraction efficacy. In general, optimization of a process could be achieved by either empirical or statistical methods; the former having limitations toward complete optimization. Unlike conventional optimization, the statistical optimization procedure allows one to take interaction of variables into consideration (Haaland, 1989).

The Response Surface Methodology (RSM) was used to point out the relationship existing between the response functions and the process variables as well as to determine the conditions of these variables to optimize the metal chelating activity of CA. Six central points were added to estimate the experimental error and to investigate

Table 4: Fit statistics for the response of $1/IC_{50}$ value of CA

Statistics	Values
SD	0.14
Mean	1.49
CV	9.40
Press	1.62
R-squared	0.9746
Adjusted R-squared	0.9509
Predicted R-squared	0.8608
Adequate precision	27.272

Table 5: Comparison of predicted and experimental values for the response variable, metal chelating activity (IC_{50}) of methanolic extract of CA (IC_{50})

					IC_{50}		
No.	Temp. (°C)	Speed (rpm)	Ratio (g:mL)	Time (h)	Predicted	Observed	Residual
1	25.16	171.17	40.38	1.32	0.1153	0.104	-0.0113
2	25.61	108.46	37.18	0.90	0.3296	0.343	0.00134
3	25.41	127.64	39.32	1.34	0.1972	0.1547	-0.0425

the suitability of the proposed model. The four independent variables were coded according to the following equation (Eq. 3) (Haaland, 1989):

$$x_i = \frac{(X_i - X)}{\Delta X_i} \qquad i = 1, 2, 3 \tag{3}$$

where, x_i and X_i are the dimensionless and the actual values of the independent variable i, X the actual value of the independent variable i at the central point and ΔX_i the step change of X_i corresponding to a unit variation of the dimensionless value.

The CA's ability as a potential ferrous ion chelator was evaluated with respect to Clioquinol in which evidence from phase 2 clinical trials suggested that clioquinol could halt cognitive decline in Alzheimer's disease, possibly owing to its ability to act as a chelator for metal ions (Di Chen *et al*., 2007). Clioquinol showed the best chelating activity with IC_{50} = 0.093 mg mL^{-1}, followed by methanolic extract at 0.26 mg mL^{-1} and IC_{50} = 0.69 mg mL^{-1} for aqueous extract. Methanol as a solvent was widely used in ethnopharmacological study because traditional practitioners believed that mostly the polar compounds were responsible for the claimed medicinal properties (Tan *et al*., 2005). As methanolic extract showed better iron chelating activity, it was used for the intended optimization process as aqueous extract had poor chelating activity.

The statistical model was developed by applying multiple regression analysis methods using the experimental data of the metal chelating activity which is given in Eq. 1. Characteristic of the constructed model was explained in Table 4 as the model adequacies were checked by R-Squared, Adjusted R-Squared, Predicted R-Squared, Adequate Precision and CV with the guide provided by Myers and Montgomery (2002) as the following: R-Squared>0.95; (Adjusted-R-Squared-Predicted-R-Squared)<0.2; CV< 10; Predicted R-Squared > 0.7; Adequate Precision > 4.

The statistical analysis indicates that the proposed model was adequate with very satisfactory values of the R-Squared. The adjusted-R^2 is adjusted for the number of terms in the model. It decreases as the number of terms in the model increases, if those additional terms do not add value to the model. Adequate precision is a signal-to-noise ratio. It compares the range of the predicted values at the design points to the average prediction error. Ratios greater than 4 indicate adequate model discrimination (Sereshti *et al*., 2009).

The graphical representations of the regression equations (Eq. 1) were displayed by the three-dimensional response surfaces and contour plots (Fig. 1-5). The main goal of RSM was to identify the optimum values of the independent variables efficiently so that the responses are maximized (Wu *et al*., 2007).

Verification experiments: Verification of experiments was performed at the predicted conditions derived from ridge analysis of RSM demonstrated that experimental values were reasonably close to the predicted values conforming the validity and adequacy of the predicted models (Table 5).

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REFERENCES

Beal, M.F., A.E. Lang and A.C. Ludolph, 2005. Neurodegenerative Diseases: Neurobiology, Pathogenesis and Therapeutics. University Press, UK, Cambridge, ISBN-13: 978-0-521-81166-8.

Cacace, J.E. and G. Mazza, 2002. Extraction of anthocyanins and other phenolics from black currants with sulfured water. J. Agric. Food Chem., 50: 5939-5946.

Cacace, J.E. and G. Mazza, 2003. Optimization of extraction of anthocyanins from black currants with aqueous ethanol. J. Food Sci., 68: 240-248.

Di Chen, C.C.Q., Y. Huanjie, R.A. Barrea, F.H. Sarkar and S. Shijie *et al*., 2007. Clioquinol, a therapeutic agent for Alzheimer's disease, has proteasome-inhibitory androgen receptor-suppressing, apoptosis-inducing and antitumor activities in human prostate cancer cells and xenografts. Cancer Res., 67: 1636-1644.

Dinis, T.C.P., V.M.C. Madeira and L.M. Almeida, 1994. Action of phenolic derivatives (acetaminophen, salicylate and 5-aminosalicylate) as inhibitors of membrane lipid peroxidation and as peroxyl radical scavengers. Arch. Biochem. Biophys., 315: 161-169.

Haaland, P.D., 1989. Experimental Design in Biotechnology. Marcel Dekker, New York, ISBN: 0-8247-7881-2.

Kabbashi, N.A., H.N. Abdurahman, S.A. Muyibi and Y.Q. Isam, 2009. Development of a wasterwater treatment system for chromium using chitosan. Int. J. Chem. Tech., 1: 44-51.

Kathy, K., 2000. Gotu kola. Better Nutr., 62: 20-20.

LeVine, S.M., J.R. Connor and H.M. Schipper, 2004. Redox-Active Metals in Neurological Disorders. New York Academy of Sciences, New York, ISBN: 1573315036.

Madamba, P.S., 2002. The response surface methodology: An application to optimize dehydration operations of selected agricultural crops. Lebensm.-Wiss. u.-Technol., 35: 584-592.

Mandel, S. and M.B.H. Youdim, 2004. Catechin polyphenols: Neurodegeneration and neuroprotection in neurodegenerative diseases. Free Radic. Biol. Med., 37: 304-317.

Mandel, S., O. Weinreb, L. Reznichenko, L. Kalfon and T. Amit, 2006. Green Tea Catechins as Brain-Permeable, Non Toxic Iron Chelators to Iron Out Iron from the Brain. In: Oxidative Stress and Neuroprotection, Parvez, H. and P. Riederer (Eds.). Springer Wien New York, New York, pp: 249-258.

Myers, R.H. and D.C. Montgomery, 2002. Response Surface Methodology: Process and Product Optimization Using Designed Experiments. 2nd Edn., Wiley, New York, ISBN: 978-0-471-41255-7.

REUTERS, 2009. 35 juta hidap Alzheimer. Utusan Malaysia. pp: 15.

Satake, T., K. Kamiya, Y. An, T. Oishi and J. Yamamoto, 2007. The anti-thrombotic active constituents from *Centella asiatica*. Biol. Pharm. Bull., 30: 935-940.

Sereshti, H., M. Karimi and S. Samadi, 2009. Application of response surface method for optimization of dispersive liquid-liquid microextraction of water-soluble components of Rosa damascena Mill. essential oil. J. Chromatogr. A, 1216: 198-204.

Shinomol, G.K. and Muralidhara, 2008. Effect of *Centella asiatica* leaf powder on oxidative markers in brain regions of prepubertal mice *in vivo* and its *in vitro* efficacy to ameliorate 3-NPA-induced oxidative stress in mitochondria. Phytomedicine, 15: 971-984.

Tan, M.L., S.F. Sulaiman, N. Najimuddin, M.R. Samian and T.S. Tengku Muhammad, 2005. Methanolic extract of *Pereskia bleo* (Kunth) DC. (Cactaceae) induces apoptosis in breast carcinoma, T47-D cell line. J. Ethnopharmacol., 96: 287-294.

Wettasinghe, M. and F. Shahidi, 1999. Evening primrose meal: A source of natural antioxidants and scavenger of hydrogen peroxide and oxygen-derived free radicals. J. Agric. Food Chem., 47: 1801-1812.

Wu, Y., S.W. Cui, J. Tang and X. Gu, 2007. Optimization of extraction process of crude polysaccharides from boat-fruited sterculia seeds by response surface methodology. Food Chem., 105: 1599-1609.

Zainol, M.K., A. Abd-Hamid, S. Yusof and R. Musec, 2003. Antioxidative activity and total phenolic compounds of leaf, root and petiole of four accessions of *Centella asiatica* (L.) Urban. Food Chem., 81: 575-581.