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Optimisation Of Extraction Conditions Of Lutein Extraction From (*Tagetes Erecta L.*) Using Response Surface Methodology

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Abstract*— Response surface methodology was used to optimize the effect of three parameters (extraction time, extraction temperature and ratio of material/ solvent) on lutein extraction content. The physicochemical characterizations and the antioxidant activity of raw material and obtained extract were investigated. Results showed that the moisture content, the lutein content and the total carotenoid content in raw material were 88.74% wb, 8.595 g/kgdb and 49%db, respectively. The highest lutein extraction content of 68.94%,db was achieved through the optimum extraction onditions of 59ºC, 47 minutes, 1/38 (w/v) for extraction temperature, extraction time and the ratio of material/solvent, respectively. In vitro free scavenging of extracted lutein was expressed by the IC50 value of 91.62 µg/ml.

Keywords—extraction; lutein; response surface methology; Tagetes erecta L.

I. INTRODUCTION

Lutein is a member of xanthophyll, a subgroup of carotenoid, a yellow orange natural pigment, which has strong anti-oxidative properties. The chemical formula of lutein is $C_{40}H_{56}O_2$, the molecular weight is 568.88 and the melting point is 190ºC. Lutein is not only contained in a variety of dark-green vegetables and fruits but also in the eye tissues and yolk. Lutein is useful to protect eyes that help preventing macular degeneration, cataract, and improving vision [1]. Since it is impossible to synthesize lutein in human body itself, the substance could be provided from food supplements, fruits, and vegetables. Lutein presents in two forms including free lutein and lutein esters with fatty acids [2-4].

Marigold (*Tagetes erecta* L.) has been globally utilized as a traditional decorative plant thanks to its color which varied from yellow and orange. Marigold flower petals are excellent sources of lutein as they contain high levels of lutein (of the order 4500 mg/lb) and no signifcant levels of other carotenoids [5]. Marigold flower petals contain lutein ester in large quantity (1-1.6%, calculated on dry weight basis (db)). In a previous study, extracts of lutein ester was found to be constituted of more than 80% of carotenoids [6]. The level of lutein in natural sources relates to their kind, variety, part of the fruit, and maturity.

Response surface methodology (RSM) has been successfully used for optimisation of biochemical extraction processes. This method enables the evaluation of the effects of several process parameters and of their interactions on the response variables [7].

The aims of this study is to optimise the conditions for lutein extraction from marigold flower. After a set of prospective test, three parameters (extraction time, extraction temperature and stirring speed) were optimised using RSM in order to improve the yields of lutein extraction. The antioxidant activity of the extract was also evaluated using DPPH (2, 2-diphenyl-1-picryl-hydrazyl-hydrate) free radical method.

II. MATERIALS AND METHOD

Materials: The marigold plant was cultivated in Tien Giang province, Viet Nam. After blooming for 60-65 days, the flowers were harvested. The orange petals were cut and eliminated the white calyx. The petals were then washed and dried for storage.

Chemical agents: All the chemicals and solvents used for extraction were purchased from Bach Khoa Company Ltd., Viet Nam.

Equipments: Memmert thermostatic waterbath WNB.14, Stuart Rotary Evaporator RE300B, Thermo Spectrophotometer GENESYS 20, Memmert INE 500 Incubator, and Lab Chroma Meter CR-400.

Lutein extraction

Orange petals were cut from marigold flowers to separate the white calyx. The petals were then be washed and dried for storage. The material was extracted with different conditions of solvent, moisture content of material, extraction temperature, extraction time and stirring speed in order to achieve the highest lutein extraction content. The extract was then filtered through a filter paper to remove insoluble substances. In order to obtain crude lutein, the extract was evaporated using Stuart Rotary Evaporator RE300B at vacuum pressure and a temperature of 50ºC, avoiding natural light to limit the ability to oxidize and decompose lutein. The crude lutein was then purified by dissolving in isopropanol at 75°C to dissolve lutein following by a filtration through membrane to remove

insoluble compounds. The filtrate was then cooled to 15°C, left overnight in the refrigerator to facilitate crystallization of the lutein. The solution was filtered over anhydrous Na_2SO_4 and dried at temperature of 50ºC in order to remove the solvent and obtain lutein extract.

Determination of total lutein extraction content

The extracted lutein was diluted to conduct optical measurement. A volume of 3.5ml of diluted solution was introduced to a cuvette of size 10mm × 10mm × 4.5mm. The mesurement was conducted at 445 nm by Thermo Spectrophotometer GENESYS 20 using ethanol as calibration [8]. Total lutein in the sample (calculated on dry weight basis (db)) is calculated according to the following formula in Eq.1:

$$\text{Total lutein content (mg/kg)} = \frac{A.V.D.10000}{2500.d.G} \quad (1)$$

Where:

A: Absorption

V: Volume of the sample (ml)

D: Dilution coefficient

2500: Average absorption coefficient of lutein 1% (w/v).

d: Thickness of the cuvette (d = 1cm)

G: Dry mass of sample (g)

Physicochemical properties characterization

TCC was determined according to the spectrophotometric method of Tran and others (2008) by UV-visible spectrophotometer at wavelength 473 nm (Thermo Spectronic, Model Genesys 20, Thermo Fisher Scientific, Waltham, Mass., U.S.A.) [9]. TSS measurement was carried out using handheld refractometers (Atago HSR-500, Atago Co., Tokyo, Japan). TSC was determined using the method of Cassano and others (2007) with modifications: centrifugation was realized at 3000rpm in 30 min with the acceleration 8500 m/s using centrifuge Hettich EBA 20 (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany), the weight of solids was determined after removing supernatant [10]. Color measurement was determined using a Minolta Chroma Meter and expressed as CIE values of L , a, and b [12]. TAA was determined by 2,2-diphenyl-1-picrylhydrazyl (DPPH) test that is modified version of Thaipong and others (2006) protocols, as presented in Mai and others [12]. The antioxidant-free radicals will transfer DPPH (1,1-diphenyl-2-picrylhydrazyl) free radicals from violet to light yellow. The free radical capture of the substance is determined by measuring the absorbance of the sample at wavelength l = 517 nm. Ascorbic acid is used as a reference material. Percentage of DPPH radical capture of research substance is calculated according to the following the Eq.2:

$$Q(\%) = \left[1 - \frac{(A - A_c)}{(A_0 - A_c)}\right] \times 100 \quad (2)$$

Where:

A is the absorbance of the solution containing the sample

A_0 is the absorption of DPPH without the sample

A_c is the absorption capacity of the solution containing the reference substance

The IC50 value is calculated using the GraphPad Prism software through a percentage of inhibitory curve (the standard curve is constructed from 6 different concentrations).

Experimental design and statistical analysis

Prospective experiments

The effect of drying time on lutein extraction content were carried out at drying time of 0, 4, 6 and 8 hours. The effect of solvent on lutein extraction content was conducted with solvents ethanol 96^0, ethanol 80^0 and ethanol 70^0. Next, the effect of the ratio of material/solvent ratio (w/v) on lutein extraction content was investigated at 1/20, 1/30, 1/40, 1/50 and 1/60 (w/v). The impact of extraction temperature on lutein extraction content was conducted at extraction temperatures of the ambiance temperature, 40^0C, 50^0C and 60^0C. The effect of extraction time was conducted at 1, 2 and 3 hours. The effect of stirring speed was evaluated at different speed of 100rpm, 300rpm, 500rpm and 600rpm.

Optimization of lutein extraction conditions using RSM (Response Surface Methodology)

After conducting the prospective experiments, the RSM coupled with central composite design (CCD) was used for

experiment design. The software JMP v. 10.0 (SAS, Cary, NC, USA) was employed to generate the experiment de-

signs, statistical analysis and regression model. The independent variables were : the ratio of material/solvent (X_1), extraction temperature (X_2) and extraction time (X_3). The experiment was designed at three levels (-1, 0,+1) for each factor. A total of 15 combinations of independent variables were realized. The response of the model, which was the lutein extraction content (in mg/kg db), was modelled following a quadratic regression model in Eq. 3:

$$Y = aX_1^2 + bX_2^2 + cX_3^2 + abX_1X_2 + acX_1X_3 + bcX_2X_3 \quad (3)$$

Where: a, b, c were the coefficients of the parameters X_1, X_2, X_3.

Statistical analysis

All the experiments were arranged in randomized form, in triplicate. The data were statistically analyzed using the Statgraphics centurion XV software, P <0.05 indicatees that the effect of the impact factors on the results was significant at 95% confidence intervals and the difference between treatments was tested by LSD method.

III. RESULTS AND DISCUSSIONS

Figure 1: Fresh marigold and dried material

Results of chemical identification of the raw material showed the appearance of tannin, phenol, alkaloids, flavonoids and glycosides in marigold petals. Quinone, resin, oxalate, terpenoid and carbohydrate compounds were not detected in the marigold petal. Fresh marigold petals have a moisture content of 88.74% (wet base). The total lutein content was 8595.2 (mg/kg db). Total ash content was 0.6797 ± 0.038%. Fresh marigold petals were orange-yellow (Fig. 1). The color indices L*, a* and b* of the fresh marigold petal were respectively 69.49; 26.69, and 52.69 measured by Lab Chroma Meter CR-400.

Effect of drying condition of raw material on lutein content

Results presented in Fig. 2 indicated that drying temperature had a significant effect on lutein extraction content. The lutetin extraction content of the samples dried at 60°C was higher than of samples dried at 75°C. When drying at 60°C, the lutein extraction content was 1643,022 (mg/kg db). Meanwhile, the sample dried at 75ºC, lutein content was 1685,689 (mg/kg db). The highest content of extraction lutein (1685.689 mg/kg db) was obtained when drying the material in 4h (Fig. 3). Single-factor ANOVA analysis showed that drying time had a significant effect on lutein content of marigold petals when compared to the fresh sample (0h). The multiple range test and LSD showed that each drying time (4h, 6h, and 8h) had a significantly different impact on the lutein content. The fresh marigold petals contain a high water content and the petals are covered with a complex cellulose-hemicellulose-pectin film [13], ethanol solvent is difficult to penetrate and dissolve lutein. With dried sample, this membrane would be destroyed that could increase the lutein extraction content.

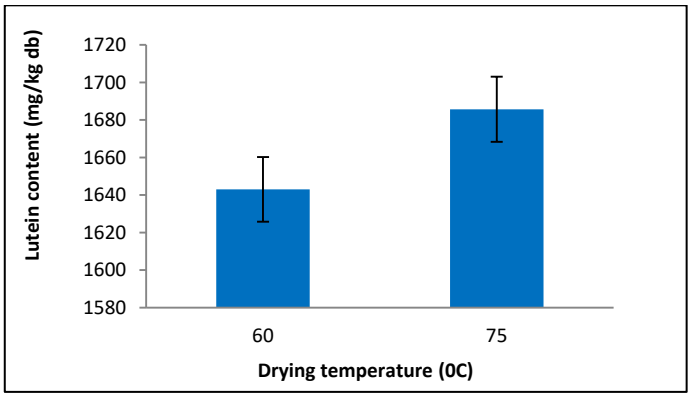


Figure 2: Effect of moisture on lutein content

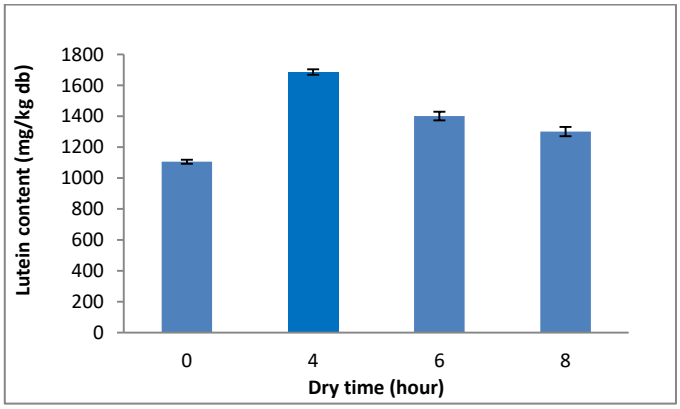


Figure 3. Effect of drying time on lutein content

Effect of solvent extraction on lutein extraction content

The lutein extraction content increased significantly with the ethanol concentration (Fig. 4). When using ethanol 70º, the lutein extraction was found to be the lowest (996. 8 mg/kg db) content. The highest lutein content (3027.733 mg / kg db) was obtained when using ethanol 96º. This is explained by a gradual decrease in polarization and an increase in the purity of the extraction solvent when reducing the ratio of water in the solvent. Single-factor ANOVA analysis showed that ethanol concentration had a significant effect on lutein extraction content at the 95% confidence level. The multiple range test and LSD presented a significant difference between the three extraction solvents (ethanol 70º, ethanol 80º and ethanol 96º).

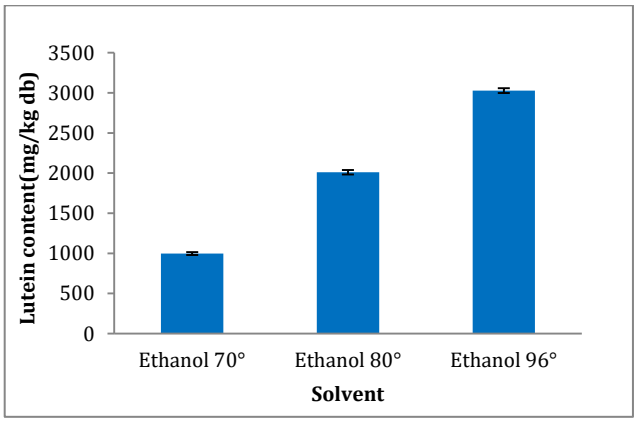


Figure 4: Effect of solvent extraction on lutein extraction yield

Effect of ratio of material/solvent extraction on lutein extraction yield

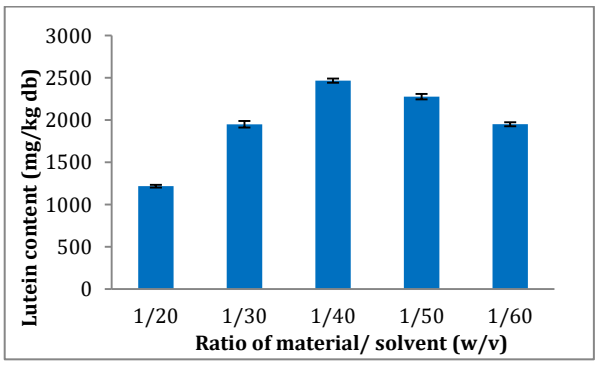


Figure 5: Effect of material ratio/ solvent on Lutein content

The extraction of the ratio of material/ solvent = 1/40 (w/v) had the highest lutein content (2466,133mg /kg db), and that of the ratio of material: solvent = 1/20 (w/v) had lowest lutein content (1217,867mg/kg db) (Fig.5). When the ratio of material/ solvent increased from 1/ 20 to 1/40 (w /v), the lutein content increased gradually however when increasing the ratio to 1/ 50 and 1/ 60 (w/v), the lutein content decreased gradually. Single-factor ANOVA analysis showed that the ratio of material/ solvent had significant effects on lutein content at the 95% confidence level. The multiple range test and LSD results showed that the lutein extraction content of the ratio of material/ solvent = 1/30 was not different compared to that of the ratio of material/solvent= 1/60. However, there was a significant difference among the content of lutein when using the ratio of mateiral/solvent of 1/20, 1/30, 1/40 and 1/50 (w/v).

Effect of extraction temperature on lutein extraction yield

Lutein extraction content changed with extraction temperature (Fig. 6). The highest lutein was extracted at 50ºC. However, when increasing extraction temperature more than 50ºC, the lutein extraction content was decreased gradually. Single-factor ANOVA analysis presented that extraction temperature had significant effect on lutein content at the 95% confidence level. The multiple range test and LSD results showed that there was a difference among extraction temperature of ambiance temperature (about 32ºC), 40ºC, 50ºC and 60ºC.

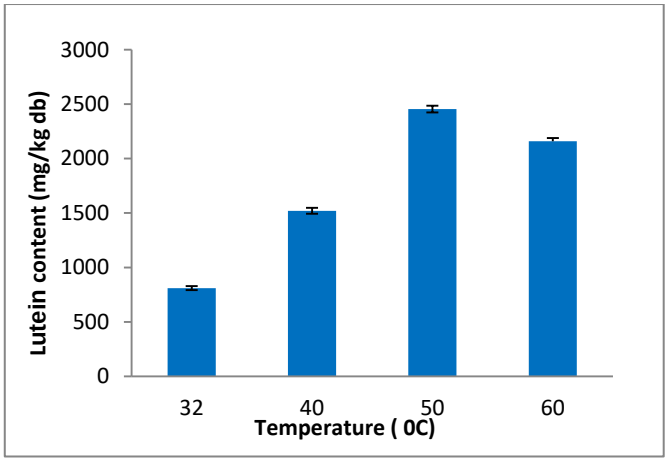


Figure 6: Effect of extraction temperature on lutein content

Effect of extraction time on lutein extraction content

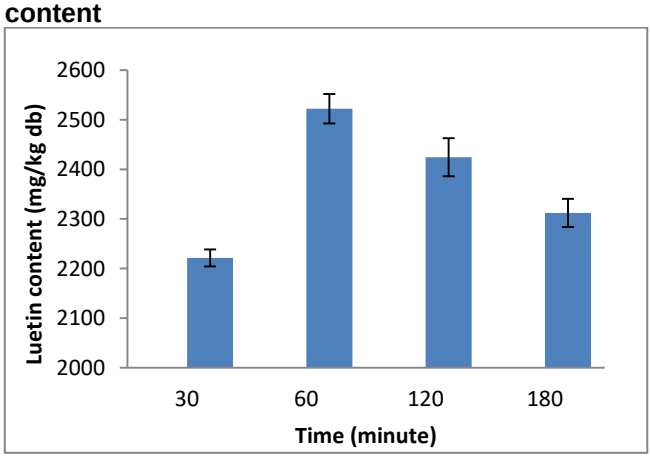


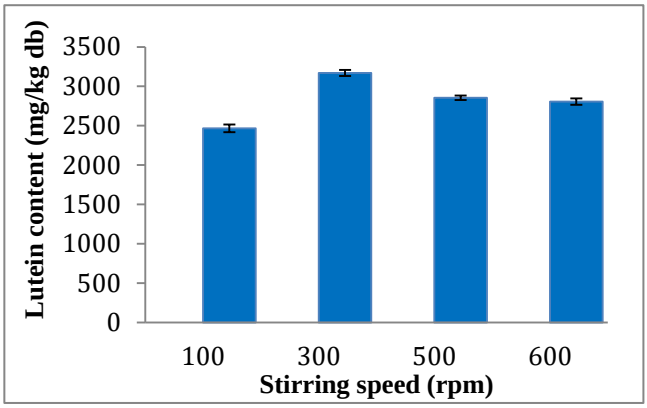
Figure 7: Effect of extraction time on lutein content

Lutein extraction content changed with extraction time (Fig. 7). The highest lutein was extracted in 60 minutes (2522.133 mg/kg db). However, when increasing extraction time (120 minute and 180 minutes), the lutein extraction content was decreased. Single-factor ANOVA analysis presented that extraction time had significant effects on lutein content at the 95% confidence level. The multiple range test and LSD results showed that there was a difference among extraction time of 30 minutes, 60 minutes, 90 minutes, and 180 minutes.

Effect of stirring speed on lutein extraction content

Lutein extraction content changed with stirring speed during extraction (Fig.8). When stirring speed increased from 100 rpm to 300 rpm, the lutein content increased and peaked at 3169.067 mg / kg db. When increasing stirring speed higher than 300 rpm caused reduce the content of lutein. Lutein content was lowest (2466.133 mg/kg db) at the stirring speed of 100rpm. Single-factor ANOVA analysis presented

that stirring speed had significant effects on lutein content at the 95% confidence level. The multiple range test and LSD results showed that there were no significant difference between yields at the speed of 500 rpm and that of 600 rpm. However, the difference was striking between the stirring speed of



100 rpm and that of 300 rpm.

Figure 8. Effect of stirring speed on lutein content

Optimization of extraction conditions by RSM

Based on the results of the proximate experiments, the optimization experiments was designed with independent variables: the ratio of material: solvent (1/50- 1/33, w/v), extraction temperature (40-60ºC) and extraction time (30-90 minutes). Each variable had three regularly spaced levels (Table 1). The CCD contains an imbedded factorial design with central points, which is augmented with a group of star points that allow the estimation of curvature [15]. The central experiment (1/40 w/v, 50^0C, 60 minutes) was performed in triplicate. Fifteen combinations of independent variables were realized.

Table 1: The central composite experimental design (in coded level of 3variables) and their levels employed for the extraction

Factorial Variable	-1	0	+1
Ratio of material: solvent (w/v)	1/50	1/40	1/33
Extraction temperature (^{0}C)	40	50	60
Extraction time (minute)	30	60	90

The variance analysis showed that the extraction temperature had a significant effect on the lutein extraction content. Two factors of extraction time and the ratio of material/solvent had no significant effect. All others first and second order terms of the three factors were not significant. The cross product coefficients were not significant, meaning that there was no significant interaction between these factors. The fitting of the quadratic regression model leaded to the following expression:

$$Y = 5286.6493 + 1335.37*X_1 - 840.1613X_1^2$$

Where X_1: extraction temperature (ºC)

The determination coefficient (RSq =0.94) indicates a good fitting quality. The value of the adjusted determination coefficient (R =0.85) was also sufficiently high to advocate a high significance of the model. Comparison of the actual and predicted values of the response for oil recovery is presented in Figure 9.

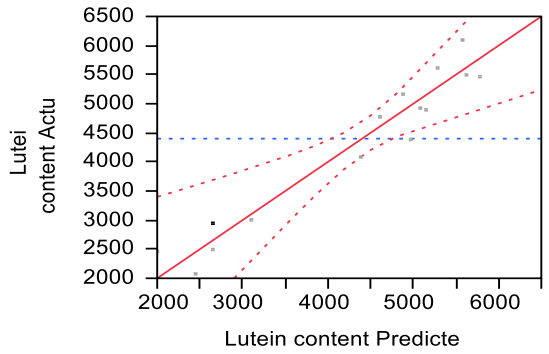


Figure 9: Comparison of predicted and actual values of lutein content

The optimization of the extraction conditions using RSM indicated that maximum lutein extraction content was obtained under the following conditions: extraction temperature of 58.94ºC, extraction time of 47.23 minutes and the ratio of material/solvent of 1/38 (w/v). The best experimental lutein content achieved was 5925.33 mg/kg db and the calculated amount of lutein extraction content with these parameters using the regression model was 5670.5 mg/kg db. This proved that this model was meaningful and suitable for lutein extraction content.

Lutein extract characterization

Results of chemical identification showed the appearance of alkaloids and glycosides in the extracted lutein. Tannin, phenol, alkaloids, quinone, resin, oxalate, terpenoid and carbohydrate compounds were not detected in the extracted lutein. Extracted lutein had the moisture content of 15.52% (wet base). The total carotenoid content was 48.5%. The color indices L*, a* and b* of the extracted lutein was respectively 53.3; 31.25, and 30.01 measured by Lab Chroma Meter CR-400. Results of microorganisms and heavy metals presented in the extract were presented in Table 2.

Table 2: Analysis result of microorganisms and heavy metals in extract product

No.	Parameter	Unit	Result	Methodology
1	Plumbum (Pb)	mg/kg	KPH (LOD=0.01)	AOAC 999.11
2	Hydrargyrum (Hg)	mg/kg	KPH (LOD=0.01)	AOAC 999.11
3	Total aerobic microorganisms	CFU/g	9.0*101	ISO 4833-1:2013
4	Escherichia Coli	CFU/g	Not detected	ISO 16649-2:2001
5	Total number of yeast spores, mold	CFU/g	Not detected	ISO 21527-2:2008
6	Salmonella	/25g	Not detected	ISO 6579-1:2017

The antioxidant capacity of the extracted lutein was presented by IC value of 91.62 ± 0.91 µg / ml compared to that of control vitamin C is 16.37 ± 1.458 µg / ml (5.7 times higher). IC50 is the concentration of the reducing product of 50% DPPH radical under conditions of determination (Fig. 10). The lower the IC50, the higher the antioxidant activity.

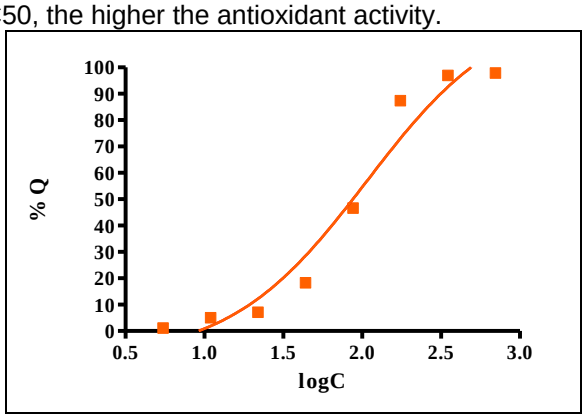


Figure 10. Percentage capture of DPPH radical scavenging by extracted lutein concentration

CONCLUSION

The optimal conditions for extracting lutein from dried marigold flowers were as follows: Extraction solvent of ethanol 96^{o} in dark and closed system, ratio of material/solvent of 1/38 (w/v), extraction time of 47 minutes, extraction temperature of 59^{o}C, and stirring speed: 300 rpm. The lutein extraction yield was about 68.94%. The extracted lutein could be used as a natural pigment in food industry thanks to its high content of lutein and antioxidant activity.

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