

Optimisation of Solid Liquid Extraction of *Orthosiphon stamineus* Leaves using Response Surface Methodology Technique

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ABSTRACT

Orthosiphon stamineus is one of the popular medicinal plants in Southeast Asia. *O. stamineus* leaves are used in numerous applications related to medicinal purposes and are believed to cure certain health conditions such as hypertension, gout and fever. The aim of this study was to investigate the effect of three parameters involved in extraction process including extraction temperature, extraction duration and solvent to solid ratio on extraction yield, antioxidant activity and referral markers of *O. stamineus* leaves. The optimisation of extraction processes was evaluated with the aid of Design-Expert software using response surface methodology (RSM). The optimum extraction parameter for *O. stamineus* leaves were recorded at the extraction temperature of 60°C, 30:1 (ml:g) solvent to solid ratio and 6 hours extraction duration with 30Wt% extract, 67 and 1 mg/L concentration of Rosmarinic acid and Sinensetin, respectively. Antioxidant activity for optimized extract is 96.56% and 91.51% of SOD and DPPH method, respectively.

Keywords: Optimization process, extraction parameter, antioxidant activity, response surface methodology, *O. stamineus*

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INTRODUCTION

Medicinal plants are promising sources for treating several diseases nowadays due to the phytochemical contents inside them. *O. stamineus* belongs to the Lamiaceae family and has several local names including “Misai

kucing” in Malaysia, “Kumis kucing” in Indonesia, and “Rau meo” in Vietnam (Awale *et al.*, 2003). *O. stamineus* has been used widely in Southeast Asia for treatment of gallstone, hepatitis, hypertension and renal calculus (Tezuka *et al.*, 2000). In Malaysia, the tea prepared from the leaves is taken as a beverage to improve health and also for the treatment of some diseases such as gout and diabetes (Akowuah *et al.*, 2004).

According to Akowuah *et al.* (2004), the separation of bioactive compounds in *O. stamineus* leaves resulted in three methoxylated flavones, namely, sinensetin (SEN), eupatorin (EUP) and 3'-hydroxy-5,6,7,4'- tetramethoxyflavone (TMF) and rosmarinic acid (RA), which are caffeic acid derivatives.

In this study, only RA and SEN were selected as referral markers because of their ability and importance. RA is one of the major phenolic acids that has been reported to possess biological activities such as antibacterial, anti-viral and antioxidant activities (Parnham & Kesselring, 1985) with International Union of Pure and Applied Chemistry (IUPAC) name of 3-(3,4-dihydroxyphenyl)-2-[(E)-3-(3,4-dihydroxyphenyl)prop-2-enoyl] oxypropanoic acid. Other than that, the antioxidant activity of RA has been reported to be better than Trolox. Trolox is a water-soluble derivative of Vitamin E with an antioxidant activity that is similar to vitamin E, chemically known as 6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid (Lu & Foo, 2002). In addition, rosmarinic acid has also been claimed as an anti-pyretic

agent and has the ability to treat fever similar to paracetamol (Yam *et al.*, 2008).

On the other hand, SEN, with IUPAC name of 2-(3,4-dimethoxyphenyl)-5,6,7-trimethoxychromen-4-one, is a flavonoid which belongs to a group of natural substances with variable phenolic structure that is present in vegetables and plant-derived products such as from fruit, grains, bark, roots, stems, and flowers (Middleton & Kandaswami, 1998). Flavonoids are potent antioxidant, free radical scavengers and metal chelators that inhibit lipid peroxidation. SEN present in *O. stamineus* leaves is unique because of a methoxy group at C-5, a rare structural feature in flavonoids (Akowuah *et al.*, 2004). It is also reported that SEN is an important flavonoid having an antioxidant property with high chemosensitising effects, and is used to synthesize multi-drug resistance (MDR) cell for anti-cancer drugs (Choi *et al.*, 2002). The skeletal structures of RA and SEN are shown in Fig.1.

Optimization can be referred as an improvement of performance of a system, process or product to obtain the optimum benefit from it (Araujo *et al.*, 1996). Traditionally, optimization in analytical chemistry was carried out using one-variable-at-a-time method. This method is carried out by changing one parameter while other parameters are kept at constant level (Bezerra *et al.*, 2008). The major disadvantage of this method is that it does not include the interactive effects among the variables studied. Another disadvantage is the increase in number of experiments

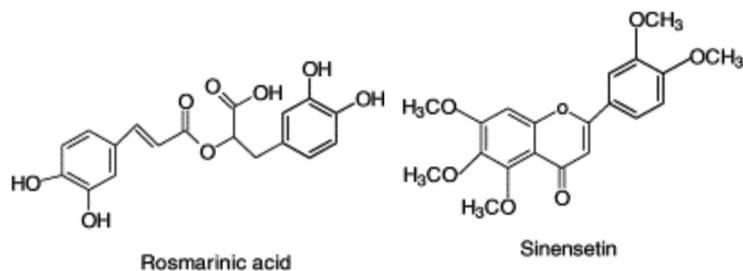


Fig. 1: Chemical skeletal structure of RA and SEN (Akowuah *et al.*, 2004)

that are necessary to conduct the research, leading to increased time and expenses required, as well as consumption of reagents and materials (Bezerra *et al.*, 2008).

There are generally four stages involved in optimisation using RSM (Bas & Boyaci, 2007). The first stage is the determination of independent variables with major effects on the system or process. The selection of the independent variables is based on the literature studied. The independent variables are usually selected to cause significant effects on Solid Liquid Extraction (SLE) such as extraction temperature, ratio of solvent to solid, and extraction duration. The second stage is selecting the experimental design according to the selected mathematical matrix. This is followed by selecting of a model as a function of independent variables and the statistical analysis evaluation of the model. Lastly, the response surface plot is used to determine the optimum points.

Yield of extract material, concentration of referral markers and antioxidant activity were strongly dependent on extraction condition. Thus, this study was aimed to investigate the effect of extraction parameters on extraction yield, antioxidant

activity, as well as the referral markers of *O. stamineus* leaves. Currently, the information on the optimum extraction of *O. stamineus* by using solvent liquid extraction (SLE) is still limited. Thus, this study will provide a good source of information for developing a new nutraceutical product based on *O. stamineus* leaves in the form of capsule and tablet.

MATERIALS AND METHODS

Materials

O. stamineus leaves were bought from a local supplier in Pulau Pinang. The leaves were kept in Raw Material Storage Room, Herbal Technology Centre, Forest Research Institute Malaysia (FRIM). Then, the samples were ground using a sieve to reduce its particle size.

Optimization of the Extraction Parameter of *O. stamineus* Leaves

The three parameters involved in this study were different extraction temperatures (40°C, 60°C and 80°C), extraction duration (4 – 8 hours) and ratio of solvent to solid (10 – 30 L/kg). The extraction was carried out using water bath (Memmert WNB

45, Germany) and water as the extraction solvent. The extraction processes were conducted in triplicate.

The extracted materials were filtered using filter paper (Whatman No. 1) and stored in a biomedical freezer at -20°C until further use. The samples were frozen dried for four days to remove the water. Yield of the extract material was calculated using the following equation (Pin *et al.*, 2010):

$$Yield (Wt\%) = \frac{W_d}{V_e} \times R_{ss} \times 100 \quad (1)$$

Where, W_d is weight of the dried extract (g), V_e is volume of the aqueous extract used for freeze-drying (ml) and R_{ss} is the solvent to solid ratio (L/kg).

Determination of RA and SEN by Using HPLC

After the freeze-dry process, the sample (10 mg) was diluted with 1 ml of water and sonicated using a sonicator (Hwashin Power Sonic Model 405, Korea) for 10 minutes. Then, the sample was filtered using a syringe filter (Whatman 0.45µm PVDF) prior to injection into HPLC to determine RA and SEN.

The HPLC system consists of Waters 600 System Controller, Waters 2996 Ultra-violet (UV) detector and equipped with Waters 717 Autosampler. Waters 2996 UV detector detects chemical compounds that pass through HPLC column and sends the data to the computer for analysis. Column oven was used to maintain the temperature of column during the analysis.

A Phenomenex Luna C18 100A column (250 mm x 4.6 mm, 5 µm particle size,

USA) was used as the stationary phase. The mobile phase was in gradient mode and comprised of 0.1% Orthophosphoric acid, H₃PO₄ and 100% HPLC grade methanol. The mobile phase combinations were selected through optimisation for better separation of compounds and shorter time. The detection wavelength chosen was 330.0 nm because the detection of RA and SEN was sensitive at that particular wavelength (Akowuah *et al.*, 2004). The experiments were conducted in triplicate and the results are presented in ppm (mg/L).

Based on the yield and HPLC analysis, prototype extract was developed using designated optimum extraction parameter, and their antioxidant activity was evaluated using Xanthine Oxidase Superoxide Scavenging Assay (SOD) and 1,2-diphenyl-2-picrylhydrazyl (DPPH) assay.

Determination of Antioxidant Activity by using Xanthine Oxidase Superoxide Scavenging Assay (SOD) Method

Stock solutions of the test samples at the concentration of 50 mg/ml were prepared by dissolving the extracts in ethanol. The reaction mixture was prepared by dissolving 0.53 g Na₂CO₃ (pH 10.2), 4.0 mg Ethylene Diamine Tetra Acetic acid (EDTA) and 2.0 mg xanthine in 0.025 mM Nitro Blue Tetrazolium (NBT) solution (100 ml of 4.1 mM/L), and adding 3.15 g Tris HCL, 0.1 g MgCl₂, 15.0 mg 5-bromo-4-chloro-3-indolyl phosphate and 34.0 mg 4- nitro blue tetrazolium chloride in 100 ml of distilled water. The mixture was kept refrigerated at 4°C.

The stock solution in 5 µl was mixed with 995 µl of the reaction mixture in a microcuvette. The microcuvette was placed in a cell holder of a spectrophotometer (Lambda 2S, Perkin Elmer, USA) and the reading was set to zero. The reaction was then initiated by the addition of 0.1 µl of XOD (1x10⁻³ U/ml). The absorbance of the resulting mixture was measured at 560 nm for two minutes.

The absorbance of the negative control was obtained by replacing the stock solution with 5 µl of the reaction mixture. SOD was used as a positive control in this assay. The percentage of inhibition was calculated as:

$$\% inhibition = \frac{Ab_c - Ab_s}{Ab_c} \times 100 \quad (2)$$

Where Ab_c and Ab_s are absorbance of the control and samples, respectively.

Determination of Antioxidant Activity by Using 1,2-diphenyl-2-picrylhydrazyl (DPPH) Method

The stock solutions of the test samples were prepared in methanol at a concentration of 0.5 mg/ml. The reaction mixture, which consisted of 4.0 ml of test solution and 1.0 ml of DPPH, was kept in a 5 ml screw cap bottle. The mixture was shaken and left at room temperature for 3 min. The absorbance of the resulting mixture was measured at 520 nm using a spectrophotometer (Lambda 2S, Perkin Elmer, USA). The absorbance of the negative control and positive control was obtained by replacing the test solution with MeOH and ascorbic acid (Vitamin C), respectively. All the reactions were

performed in triplicates. The percentage of inhibition was calculated as:

$$\% inhibition = \frac{Ab_{c,-ve} - Ab_s}{Ab_{c,-ve} - Ab_{c,+ve}} \times 100 \quad (3)$$

Where Ab_s is absorbance of the test samples, while $Ab_{c,-ve}$ and $Ab_{c,+ve}$ are absorbance of the negative and positive control, respectively.

Experimental Design

The extraction parameters were optimized using response surface methodology (RSM). A central composite design (CCD) was employed in this regard. Ratio of solvent to solid (X_1), and extraction duration (X_2) were chosen for the independent variables. Three experiments were carried out for each experimental design point and the mean values were stated as observed responses. The mathematical modelling model corresponding to the composite design is:

$$Y = B_0 + B_1X_1 + B_2X_2 + B_{12}X_1X_2 + B_{11}X_1^2 + B_{22}X_2^2 \quad (4)$$

where Y is the predicted response variable, B_0 is the regression coefficient of intercept term, B_1 and B_2 are linear regression coefficients, B_{11} and B_{22} are squared regression coefficients, and B_{12} is the interaction regression coefficient.

The analysis of variance (ANOVA) was used to evaluate the significance of each variable on the resulted model. The significance was determined statistically by computing the F-value and p-value ($p < 0.05$). The adequacy of the polynomial

model to predict the experiment was determined with correlation coefficient, R^2 . The experimental design using CCD is shown in Table 1.

TABLE 1
Central-composite experimental design of the independent variables

Experiment	Independent variables	
	X ₁	X ₂
1	10.00	4.00
2	30.00	4.00
3	10.00	8.00
4	30.00	8.00
5	10.00	6.00
6	34.14	6.00
7	20.00	3.17
8	20.00	8.83
9	20.00	6.00

RESULTS AND DISCUSSION

Effects of Extraction Temperature

Fig.2 shows the HPLC quantification analysis, whereby the results indicate that the concentration of RA increased from 40 ppm to 140 ppm as the temperature increased from 40°C to 80°C. This could explain that RA is a thermal-stable polyphenol that can withstand a high temperature (80°C). On the other hand, Fig.3 shows the contradicted results for SEN, whereby its concentration started to decrease from 0.6 ppm to 0.3 ppm as the temperature increased from 60°C to 80°C. Fig.4, Fig.5 and Fig.6 represent the HPLC chromatogram for RA and SEN at the temperatures of 40°C, 60°C and 80°C, respectively. It can be deduced that concentration of RA was highest at 80°C but SEN started to denature at

the same temperature. Meanwhile, the concentration of RA was higher compared to SEN because RA has four hydroxyl and a carboxylic group, which could effectively form hydrogen-bonding with the water, while SEN has no hydroxyl group at all. This enhances the solubility of RA in the water and results in a higher recovery in the extract compared to SEN.

Based on the figures, the most suitable extraction temperature of *O. stamineus* leaves in this study was 60°C. Akowuah and Zhari (2010) reported that the recovery of SEN from *O. stamineus* leaves extract decreased with the increasing extraction temperature above 60°C because of degradation. A similar finding was also reported; increasing the temperature of the extraction would increase the concentration of RA but decreased the concentration of SEN (Sriyana *et al.*, 2011). Silva *et al.* (2007) reported that temperature was also the most important parameter in extraction of *Inga edulis* leaves because higher temperature increased the solubility and diffusion coefficient of the solute, allowing higher yield and extraction rate. This principle is also applicable in the extraction of *O. stamineus* leaves.

Effects of Solvent to Solid Ratio on Extraction Yield

In order to select the optimum ratio of solvent to solid (X₁) and extraction duration (X₂) towards yield (Y), the quadratic model was solved using RSM and given as:

$$Y = 29.66 + 1.02X_1 + 3.86X_2 + 2.59X_1X_2 + 1.02X_1^2 + 1.61X_2^2 \quad (5)$$

TABLE 2
Turkey test for extraction time and extraction yield

Test	Time	Group a	Group b	Group c
Turkey	3 hours 30 minutes	26.94 Wt %		
	4 hours		29.67Wt%	
	6 hours			31.83Wt%
	8 hours			31.90 Wt%
	8 hours 30 minutes			39.36Wt%

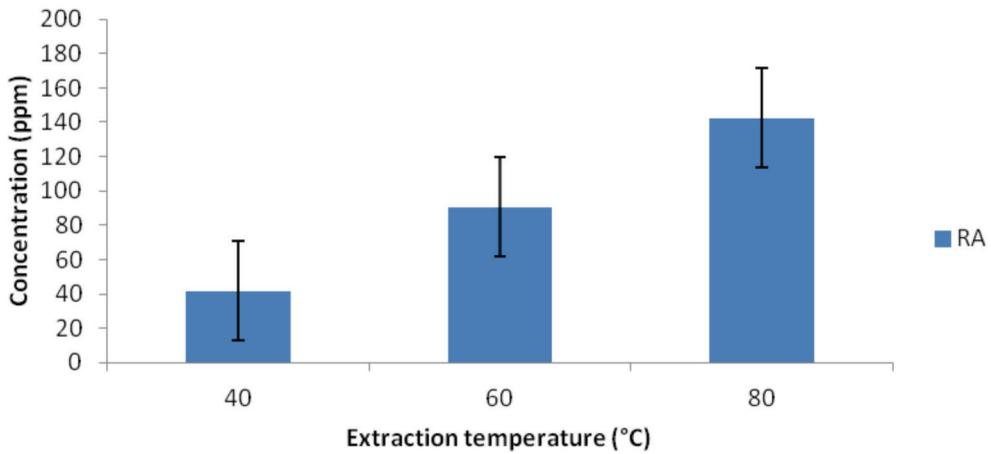


Fig.2: Concentration of RA at different extraction temperatures

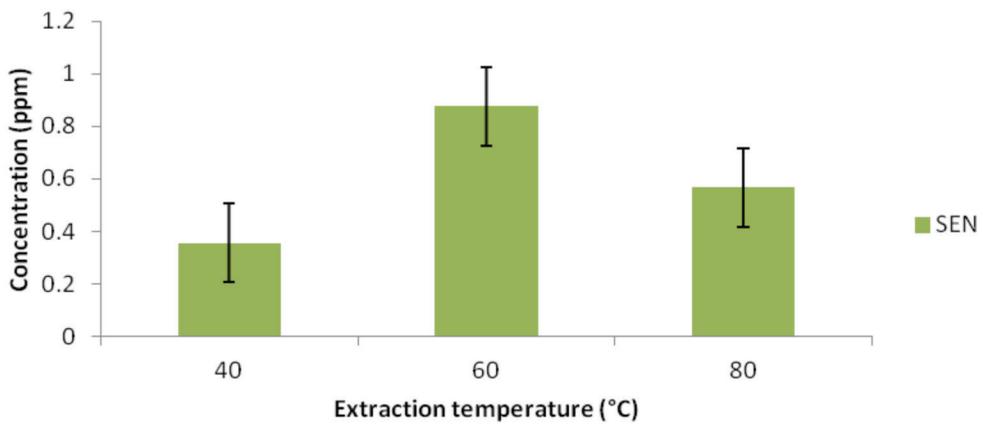


Fig.3: Concentration of SEN at different extraction temperatures

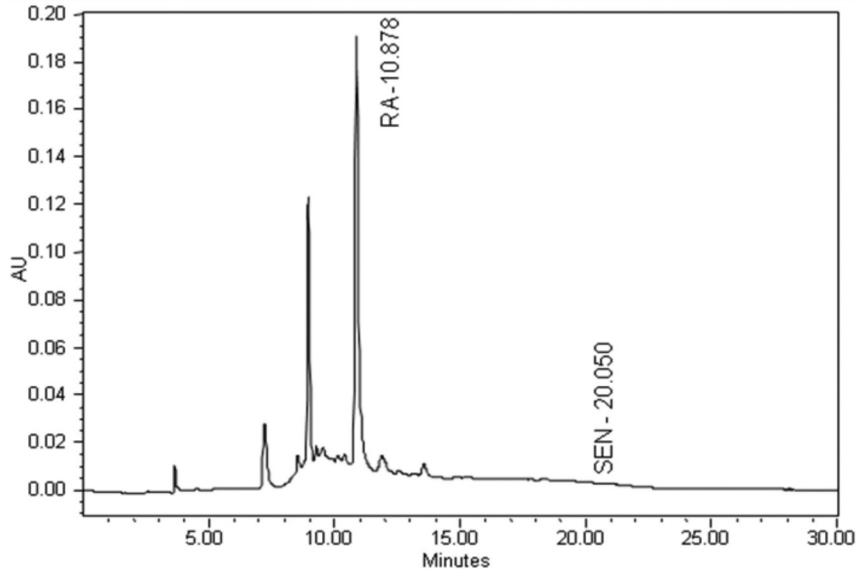


Fig.4: HPLC chromatogram for SLE of *O. stamineus* at 40°C

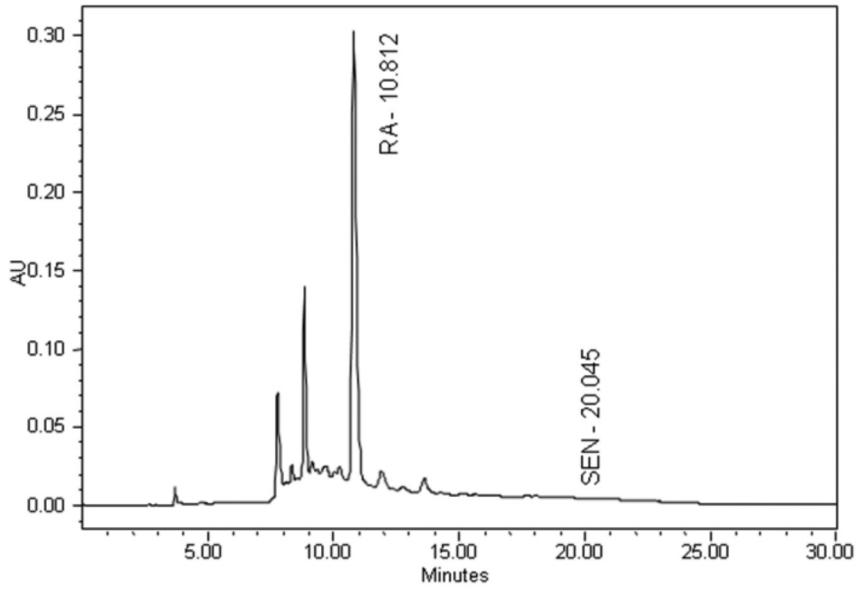


Fig.5: HPLC chromatogram for SLE of *O. stamineus* at 60°C

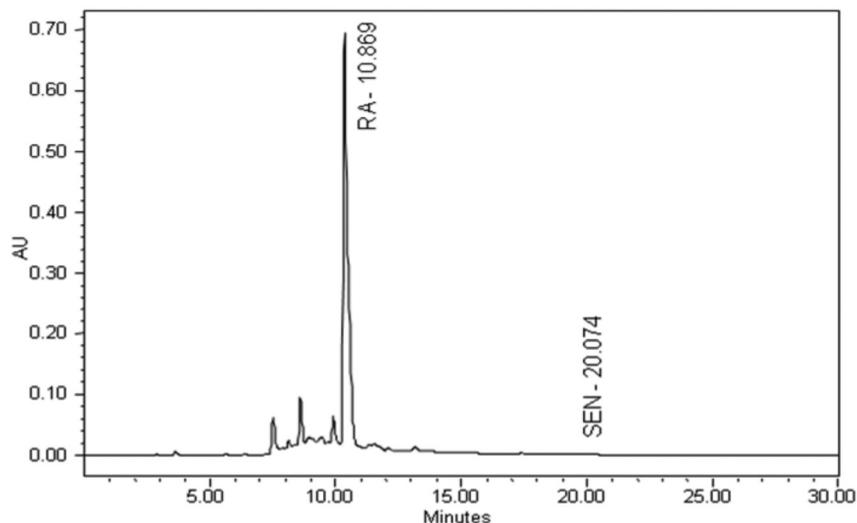


Fig.6: HPLC chromatogram for SLE of *O. stamineus* at 80°C

The model gave a satisfactory fit with the experimental data because R^2 value is 0.9725. This means that the model could explain about 97% of the total variability within the studied range.

Fig.7 shows the three-dimensional response surface graph plotted to illustrate individual and interactive effects of the independent variables on the yield of *O. stamineus* leaves extract. Based on the graph, the extraction yield was found to increase with the increase in extraction duration and ratio of solvent to solid.

Optimization Process

From the RSM analysis, the maximum extraction yield can be obtained by solving the first derivatives of Equation 4, as follows:

$$\frac{\partial Y}{\partial X_1} = 1.02 + 2.59X_2 + 2.04X_1 = 0 \quad (6)$$

$$\frac{\partial Y}{\partial X_2} = 3.86 + 2.59X_1 + 3.22X_2 = 0 \quad (7)$$

The solution of the above equations led to the maximum yield of 39.36 Wt%, with ratio of solvent to solid of 30:1 (ml:g) and the extraction duration of 8 hours and 30 minutes. However, using the SPSS analysis from Table 2, there are no significant differences in terms of the extraction yield between 6 and 8 hours extraction duration. Hence, six hours is selected as the extraction duration for *O. stamineus* leaves. The use of excessive time would lead to the increase of cost for operation. Yin *et al.* (2010) also reported that although the concentration of total phenolic content was highest at 120 min, due to economic aspect, 80 min was selected as the best extraction duration for *Morinda citrifolia*. Besides, after 8 hours of extraction duration, the amounts of RA and SEN decreased, as shown in Table 3.

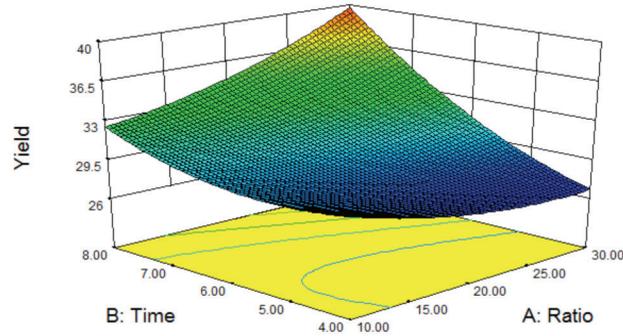


Fig.7: A 3-Dimensional response surface graph between ratio and time towards yield

TABLE 3
Concentration of RA and SEN for different extraction time

Parameter	Yield (Wt%)	RA (ppm)	SEN (ppm)
Time = 8 hours 30 min	39.36	48.67	0.66
Time = 6 hours	30.49	67.09	1.43

Based on those considerations, the optimum extraction conditions for *O. stamineus* leaves are as follows: 60°C of extraction temperature, 6 hours of extraction duration and 30 L/kg ratio of solvent to solid. Although the yield was decreased by 9% (Table 3), both RA and SEN were maintained at that condition. The concentrations of RA and SEN were determined to be 67 mg/L and 1 mg/L, respectively.

Antioxidant activity

Based on the optimised parameter obtained from this study, an antioxidant activity was evaluated and the results are tabulated in Table 4. The results showed a high antioxidant activity (above 70% inhibition) for both SOD and DPPH assays. Akowuah

et al. (2004) also reported that 84.2% of antioxidant activity was evaluated by using DPPH assays. Thus, this study has provided preliminary data for prototype extracts towards the development of nutraceutical product based on *O. stamineus*.

TABLE 4
Antioxidant activity of prototype extract of *O. stamineus* leaves

No	Sample	% inhibition	
		SOD	DPPH
1	OS R1	96.44	89.97
2	OS R2	96.71	92.20
3	OS R3	96.54	92.37

CONCLUSION

Based on the findings of this present study, it was concluded that when the extraction temperature increased, the yield of RA also increased. However, it will decrease the yield of SEN. In order to to maintain both at the optimum value, 60 °C was selected as suitable temperature.

The best extraction duration was found to be at 6 hours since at 8 hours and above of extraction duration, both RA and SEN were denatured.

The optimum ratio of solvent to solid was selected to be 30 L/kg since the results have shown high values of extraction yield and concentration of RA and SEN at that ratio.

By using this optimum parameters, the concentration of RA and SEN were yield about 67 mg/L and 1 mg/L, respectively, with 31Wt% yield extract.

The antioxidant activity of SOD and DPPH was shown as 97% and 92% inhibition, respectively. Finally, the results from this study can be used to formulate a new nutraceutical product of *O. stamineus* in the form of capsule and tablet.

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