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Full Length Research Paper

Evaluation to the antioxidant activity of total flavonoids extract from *Syzygium jambos* seeds and optimization by response surface methodology

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The purpose of this work was to assess the antioxidant activity of total flavonoids extract from *Syzygium jambos* seeds (TFSJ). Response surface methodology was employed to optimize the main extraction conditions including extraction time, ethanol concentration and solid-liquid ratio. The effect of TFSJ on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, 2,2-azino-bis(3-ethyl-benzthiazoline-6-sulfonic acid) diammonium salt (ABTS) radical scavenging and hydroxyl radical (HO•) scavenging were studied. We found that under the conditions of extraction time 32.27 min, solvent concentration 52.01% and solid-liquid ratio to be 27.32:1, TFSJ possesses considerable amounts of flavonoids of 11.4330 mg/g rutin equivalent of extract. The effect of this extract in scavenging activity of ABTS and hydroxyl radical activity was better than that of rutin. However, the effect of TFSJ in free radical scavenging of DPPH was not as good as that of rutin. In conclusion, TFSJ possess potent antioxidant and free radical scavenging activities. These antioxidant activities could contribute, at least in part, to the traditionally claimed therapeutic benefits of *S. jambos* seeds.

Key words: Total flavonoids, antioxidant activity, response surface methodology.

INTRODUCTION

Syzygium jambos Alston (*Eugenia jambos*) is a plant from the Myrtaceae family, originated in tropical Asia, specifically India. *S. jambos* seeds are used by the village people to treat illnesses caused by bacterial, fungal and viral pathogens. The seed extract of *S. jambos* was used to treat cold, cough, fever and skin problems such as rashes and the mouth, throat, intestines and genitor-urinary tract ulcers (infected by *Candida albicans*). The seeds are sweet, astringent to the bowels and good for diabetes.

Due to the popular use of *S. jambos* seeds to assist in the treatment of skin disease and diabetes, the antioxidant properties of extracts from different parts of the plant were evaluated in recent years. Numerous isolated plant constituents and crude extracts from fruits and vegetables have been recognized to possess beneficial effects against free radicals in biological systems as natural antioxidants (Jiang et al., 2011). For example, the seed kernel of the *Syzygium cumini* fruits showed high activity against the superoxide anion and hydroxyl radical compared to standards, such as catechin and trolox (Benherlal and Arumughan, 2007). In addition, *S. cumini* fruit skin showed a significant correlation existed between concentration of the extract and percent-tage inhibition of free radicals or percentage inhibition of lipid peroxidation (Banerjee et al., 2005). Compared to other fruits, three anthocyanins from *S. cumini* fruit peels were identified by HPLC–ESI–MS and evaluated for their antioxidant efficacy (Veigas et al., 2007). These beneficial effects are most probably related to the presence of bioactive compounds, such as carotenoids and phenolic compounds. The aim of this study was to determinate the total flavornoids and optimization for ultrasound-assisted extraction of *S. jambos* seeds by response surface methodology (RSM), and the evaluation of the antioxidant capacity by DPPH free radical scavenging, ABTS and hydroxyl radicals (HO•) of ethanol extracts of the plant.

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Table 1. The level of form factors.

Factor	Extraction time (X_1)	Solvent concentration (X_2)	Solid-liquid ratio (X_3)
-1	15	40	20
0	30	60	30
1	45	80	40

MATERIALS AND METHODS

Plant materials and instruments

S. jambos seeds was collected and identified by Professor Wang ZY, College of Life Science and Biopharmacology, Guangdong Pharmaceutical University, Guangdong province, China. In order to avoid degradation, the air-dried plant material was ground just before extraction.

BL-2000S Electronic Balance (SETEA Co., Ltd.), KQ-600DE Nc ultrasonic apparatus (Kunshan ultrasonic machine company), PE lambda -35 UV spectrophotometer (Perkin Elmer Co., Ltd.), and MA-110 electronic scale (precision 0.1 mg, Shanghai Scale Instrument company) were used in this study.

Chemicals and reagents

2,2-diphenyl-1-picryl-hydrazyl (DPPH), 2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulphonic acid) (ABTS) and crocus were obtained from Sigma-Aldrich, Germany. Ascorbic acid was purchased from Sigma Chemical Co. (St Louis, MO, USA). Water was purified using a Milli-Q water purification system (Milipore, USA). Standards of rutin were purchased from China pharmaceutical and biological products inspection. All other chemicals and solvents used for extraction in this study were of analytical grade and obtained from Tianjin Reagent Company (Tianjin, China).

Extraction of total flavonoids from *S. jambos*

S. jambos seeds crushed, with 24 mesh screen. The dried powder 5.0 g was added solvent (ethanol concentration 40, 60 and 80%) in proportion (solid-liquid ratio 20:1, 30:1, 40:1) and extracted with ultrasonic wave (15, 30 and 45 min) at 50 kHz. The filtrate was collected and the residue was extracted again as the protocol above. Then, the filtered solution was stored at 4°C for further analysis.

Determination of total flavonoid content

The total flavonoid content of the extract was determined by the method described in the Chinese Pharmacopoeia (Chinese Pharmacopoeia Committee, 2005). The extraction and different concentrations of the rutin standards solution were diluted appropriately and mixed with 1 ml $NaNO_2$ (5%). After standing for 6 min, 1 ml of 10% $AlCl_3$ and 10 ml of NaOH (1 M) were added to the mixture. The mixture was adjusted to 25 ml with distilled water and allowed to rest for 15 min. The absorbance (A) was measured at 510 nm, with distilled water as a blank control. Rutin was used as a reference standard and the total flavonoid content was expressed as rutin equivalents (RE, 1 g/mg extract). All determinations were performed in triplicate.

Optimization of total flavonoid by Box-Behnken design for RSM

In order to obtain suitable extraction conditions for *S. jambos*, Box-Behnken designs (BBD, Design Expert software, Trial Version 8.0.4, Stat-Ease Inc., Minneapolis, MN, USA) was applied to experimental design, data analysis and model building. Based on the preliminary tests, a total of 17 runs from BBD were employed to optimize the main extraction conditions including extraction time (X_1), ethanol concentration (X_2) and solid-liquid ratio (X_3) as Table 1 shows.

Antioxidant activity of total flavonoid from *S. jambos*

Scavenging capacity on DPPH radical

The free radical scavenging activity of each extract and ascorbic acid (control) were determined based on their ability to react with the stable DPPH free radical. In brief, 2.5 ml of the extract (12.5 to 500 µg/ml, dissolved in 95% ethanol) was added to 2.5 ml of DPPH (250 µg/ml, dissolved in 95% ethanol). The mixture was strongly shaken and maintained at room temperature for 30 min in the darkness. The absorbance was measured using a spectrophotometer (Lambda 35 UV-visible, Perkin Elmer) at 517 nm against a blank.

$$DPPH \quad scavenging\ effect(\%) = \frac{A_D - (A_H - A_0)}{A_D} \times 100$$

where A_D is A_{517} of DPPH without sample; A_H is A_{517} of sample and DPPH, and A_0 is A_{517} of sample without DPPH.

ABTS radicals scavenging assay

The antioxidant activity was determined by ABTS radical cation described by Fan et al. (2009), with some modifications. ABTS radical cation was produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulfate and the mixture was allowed to stand in the dark at room temperature for 16 h. In the moment of use, the ABTS solution was diluted with ethanol to an absorbance of 0.70±0.02 at 734 nm. 0.3 ml of each sample with various concentrations (6.25 to 150 µg/ml) were added to 3.0 ml of ABTS solution and mixed vigorously. The reaction mixture was allowed to stand at room temperature for 6 min and the absorbance at 734 nm was immediately recorded. The ABTS scavenging effect was calculated as follows:

$$ABTS \quad scavenging\ effect(\%) = \frac{A_t - (A_s - A_0)}{A_t} \times 100$$

Table 2. Results of response surface methodology.

No.	X_1	X_2	X_3	*Y(%)*
1	1	0	-1	11.17207
2	0	0	0	11.38886
3	1	0	1	10.90852
4	-1	1	0	11.08044
5	0	0	0	11.38816
6	0	-1	-1	10.77944
7	-1	0	-1	11.2168
8	1	-1	0	10.84545
9	-1	0	1	11.0131
10	0	-1	1	10.50112
11	1	1	0	11.13224
12	0	1	1	10.7604
13	0	0	0	11.38599
14	0	0	0	11.38711
15	-1	-1	0	11.11173
16	0	1	-1	11.18621
17	0	0	0	11.38494

where A_t is A_{734} of ABTS without sample, A_s is A_{734} of sample and ABTS, and A_0 is A_{734} of sample without ABTS.

Hydroxyl radical scavenging assay

The hydroxyl radical scavenging activity of each extract was measured according to the method brought forward (Wang et al., 2008) with some modifications. Different concentration (12 to 125 µg/ml) samples were incubated with 2.0 mM EDTA-Fe (0.5 ml), 3% H_2O_2 (1.0 ml) and 360 µg/ml crocus in 4.5 ml sodium phosphate buffer (150.0 mM, pH 7.4) for 30 min at 37°C, and hydroxyl radical was detected by monitoring absorbance at 520 nm. The hydroxyl radical scavenging effect was calculated as follows:

$$hydroxyl\quad radical\quad scavenging\quad effect\,(\%) = \frac{A_C - A_S}{A_C} \times 100$$

where A_S is A_{520} of sample and A_C is A_{520} of control. In the control, sample was substituted with distilled water, and sodium phosphate buffer replaced H_2O_2.

Statistical analysis

All experiments data in tables and figures represent mean values±standard deviation (n = 3). Results were evaluated for statistical significance using one-way ANOVA by SPSS V.13 (SPSS Inc., Chicago, USA). The confidence level for statistical significance was set at a probability value of 0.05. The response obtained from each set of experimental design was subjected to multiple non-linear regressions using the Design Expert software, Trial Version 8.0.4. The quality of the fit of the polynomial model equation expressed by the coefficient was checked using *F*-test and *p*-value.

RESULTS AND DISCUSSION

Total flavonoids content (TFSJ)

Calibration curves were constructed by linear regression of total flavonoids content (*Y*), versus the concentration (*x*). For linearity validation, rutin standard solutions at a concentration range of 0.036 to 2.839 mg/L, $Y = 2185.22X_1 + 3.18$ ($r = 0.9999$). The total flavonoid content of *S. jambos* extraction was expressed as rutin equivalents in mg/g of extracts. The extracts contained 11.097±0.265 mg/g total flavonoids as shown in Table 2. Because flavonoids are responsible for antioxidant activity, the high amount of total flavonoids in the extract suggests that the extract possesses an antioxidant activity *in vitro* (Lotito and Frei, 2004). Rutin is a flavonol glycoside plant metabolite with anti-oxidative, anti-inflammatory and anti-carcinogenic effects. Rutin can also reduce the fragility of blood vessels found in haemorrhagic disease and hypertension in humans (Sun et al., 2011).

Optimization of RSM

Box-Behnken design for multivariate optimization is a class of rotatable or nearly rotatable second-order designs based on three-level incomplete factorial designs (Li et al., 2011). The results indicated that the model used to fit response variable was significant ($p < 0.0001$) and adequate to represent the relationship between the response and the independent variables (Ballard et al.,

Table 3. Analysis of variance (ANOVA) for the experimental results.

Factor	Sum of square	Mean square	df	*F*-value	Prob.>*F*-value
Model	1.0967	0.1219	9	29.7987	< 0.0001
X_1	0.1062	0.1062	1	25.9607	0.0014
X_2	0.0165	0.0165	1	4.0456	0.0842
X_3	0.1715	0.1715	1	41.9445	0.0003
$X_1 X_2$	0.0253	0.0253	1	6.1856	0.0418
$X_1 X_3$	0.0054	0.0054	1	1.3299	0.2867
X_2X_3	0.0009	0.0009	1	0.2190	0.6540
X_1^2	0.3986	0.3986	1	97.4828	< 0.0001
X_2^2	0.0057	0.0057	1	1.3989	0.2755
X_3^2	0.3127	0.3127	1	76.4782	< 0.0001
Lack of fit	0.0286	0.0095	3	3784.7918	< 0.0001
Error	0.0000	0.0000	4		
Total	1.1253		16		

2009; Pierozan et al., 2009). The *F*-test suggested that the model had a very high model *F*-value (F = 29.8), indicating that this model was highly significant. R^2 adj (adjusted determination coefficient) is the correlation measure for testing the goodness-of-fit of the regression equation (Yang et al., 2009). The R^2 value of this model is 0.9746, which indicates that only 2.54% of the total variations were not explained by the model. Meanwhile, a relatively lower value of coefficient of variation (*CV*=0.58) showed a better precision and reliability of the experiments carried out (Thana et al., 2008).

It can be seen in Table 3 that extraction yield was affected most significantly by solid-liquid ratio (X_3, p = 0.0003), followed by extraction time (X_1, p = 0.0014) and solvent concentration (X_2, p = 0.0842). It was evident that two quadratic parameters (X_1^2, X_3^2) and one interaction parameters (X_1X_2) were significant at the level of p < 0.0001 or p < 0.05. The predicted response Y for the yield of extraction could be expressed by the following second-order polynomial equation in term of coded values: $Y = 11.39 + 0.12 X_1 - 0.045X_2 - 0.15X_3 + 0.080X_1X_2 - 0.037X_1X_3 - 0.015X_2X_3 - 0.31 X_1^2 - 0.037X_2^2 - 0.27 X_3^2$.

The regression equation was graphically represented by 3D response surface and 2D contour plots (Li et al., 2011). From 3D response surface curves and contour plots shown in Figures 1 to 3, the effect of the independent variables and their mutual interaction on the extraction yield can be seen. Response surfaces were plotted to study the effects of parameters and their interactions on extraction yield. Figure 1 is the response surface and contour plot showing the effect of extraction time and solvent concentration on the response at the fixed value of the ratio of solid-liquid to material. It can be seen that by increasing the extraction time, the extraction yield increased as well, reached a maximum value while the further increase of extraction time had slightly effect. Figure 2 depicts the interaction effect of extraction time and ratio of solid-liquid on the response at the fixed value of solvent concentration. The increase of solid-liquid ratio can significantly enhance the response, and then a maximum response was obtained, and beyond this level, no obvious increase was observed. Figure 3 describes the interaction effect of solvent concentration and solid-liquid ratio on the response at the fixed value of extraction time. In this study, the aim of optimization was to find the conditions which gave the maximum extraction yield in *S. jambos*. The software predicted the optimum extraction time and solvent concentration and solid-liquid ratio to be 32.27 min, 52.01%, 27.32:1. Under these conditions, the extraction yield was 11.4244 mg/g. To test validity of response surface analysis method, the extraction was carried out under the proposed conditions and the extraction yield was 11.4330 mg/g (n = 3). The good correlation between these results confirmed that the response model was adequate to reflect the expected optimization.

Antioxidant activities analysis

Effect of scavenging DPPH radicals

DPPH radical is a widely used method to evaluate the free radical scavenging ability of natural compounds (Barreca et al., 2011). In the test, the antioxidants were able to reduce the stable DPPH radical and the absorbance at 517 nm. The effect of antioxidants on DPPH radical scavenging was conceived to be due to their proton-donating ability. Therefore, the antioxidant activity of a substance can be expressed as its ability in scavenging the DPPH free radical. Our results found that both TFSJ and rutin were effective at reducing the stable radical DPPH to the yellow-coloured diphenylpicrylhydrazine, indicating that these extracts are active in DPPH radical scavenging (Figure 4). TFSJ had

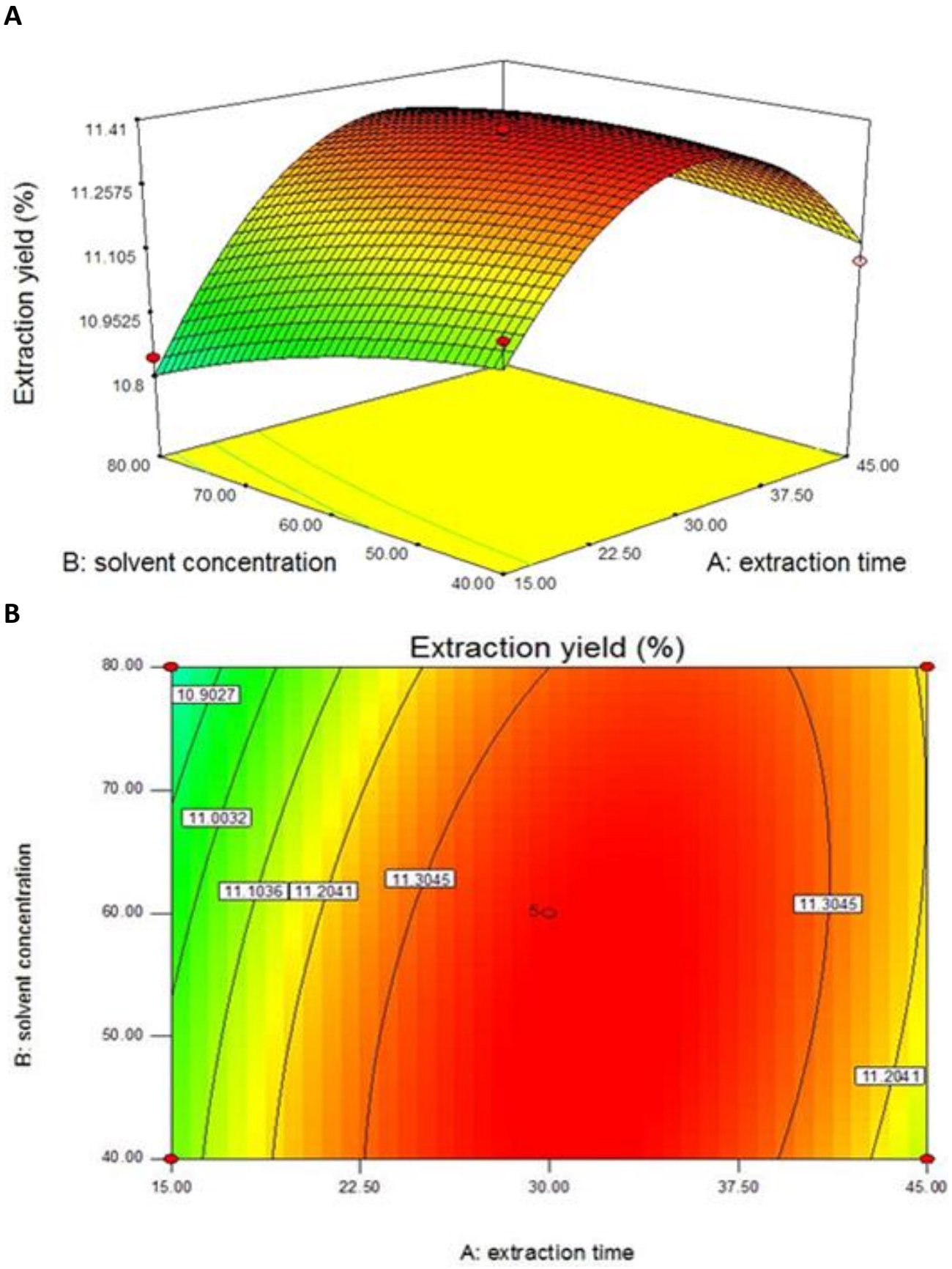

Figure 1. Response surface plot and contour plot of extraction time and solvent concentration on extraction yield.

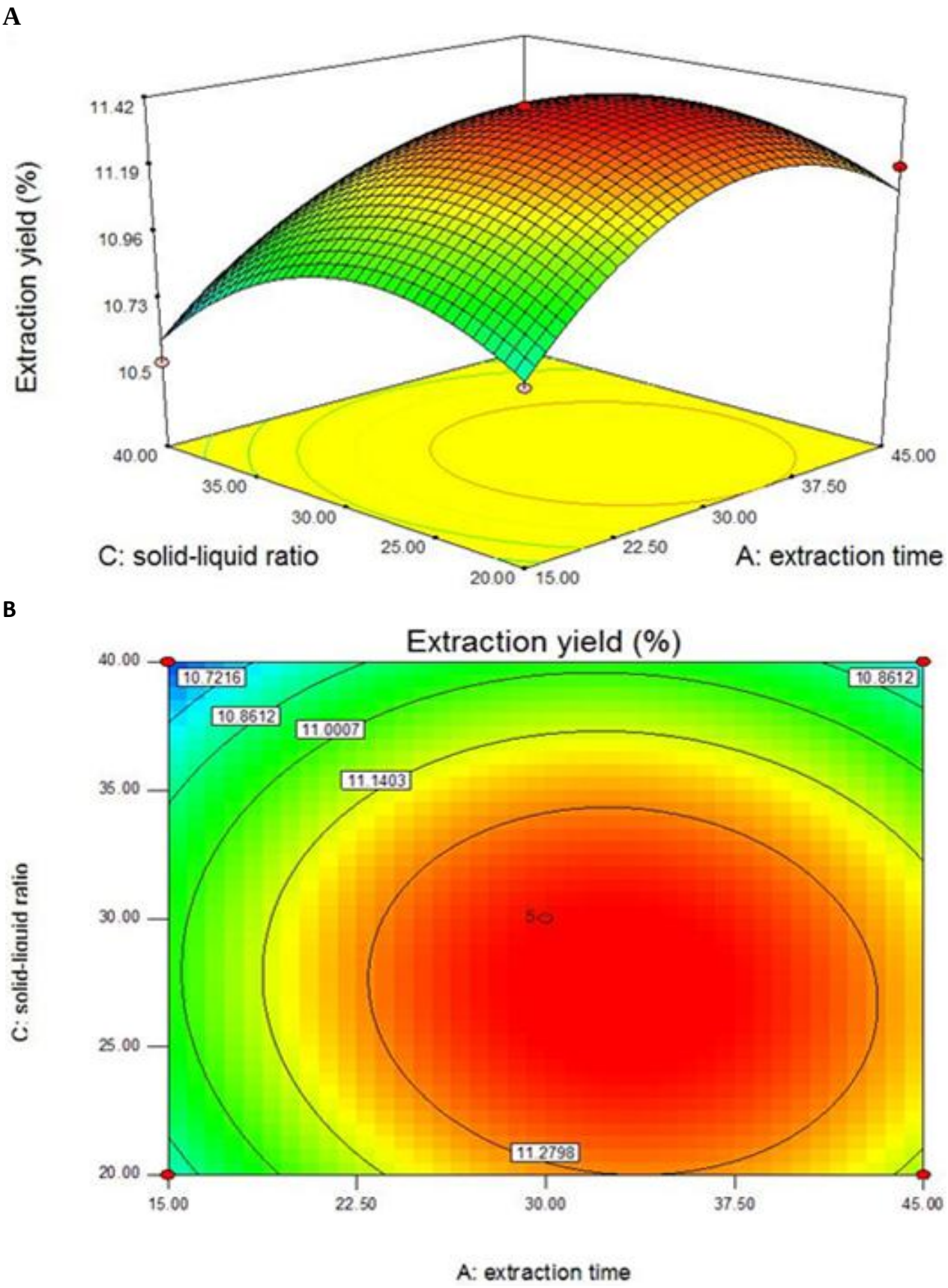

Figure 2. Response surface plot and contour plot of extraction time and solid-liquid ratio on extraction yield.

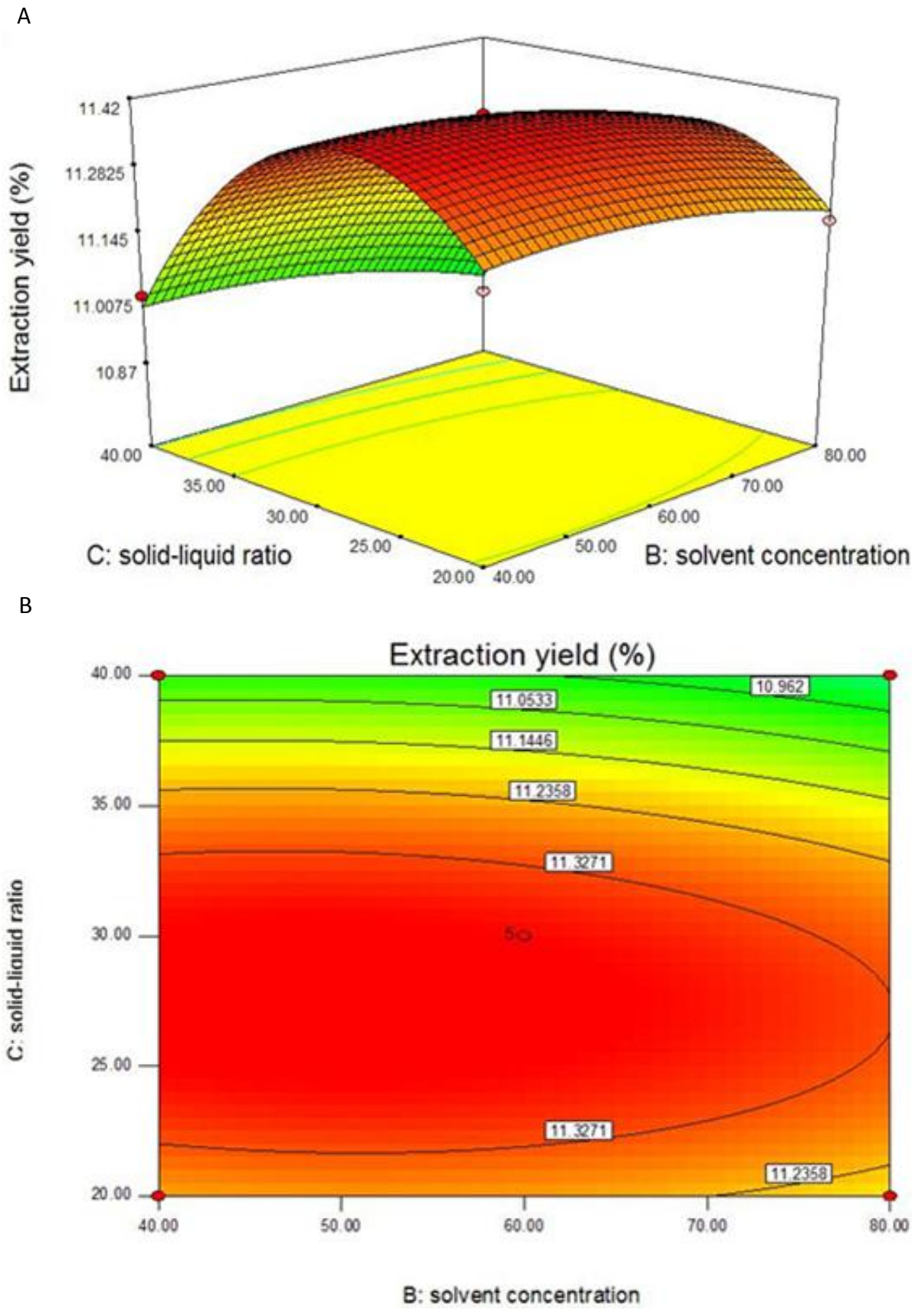

Figure 3. Response surface plot and contour plot of solvent concentration and solid-liquid ratio on extraction yield.

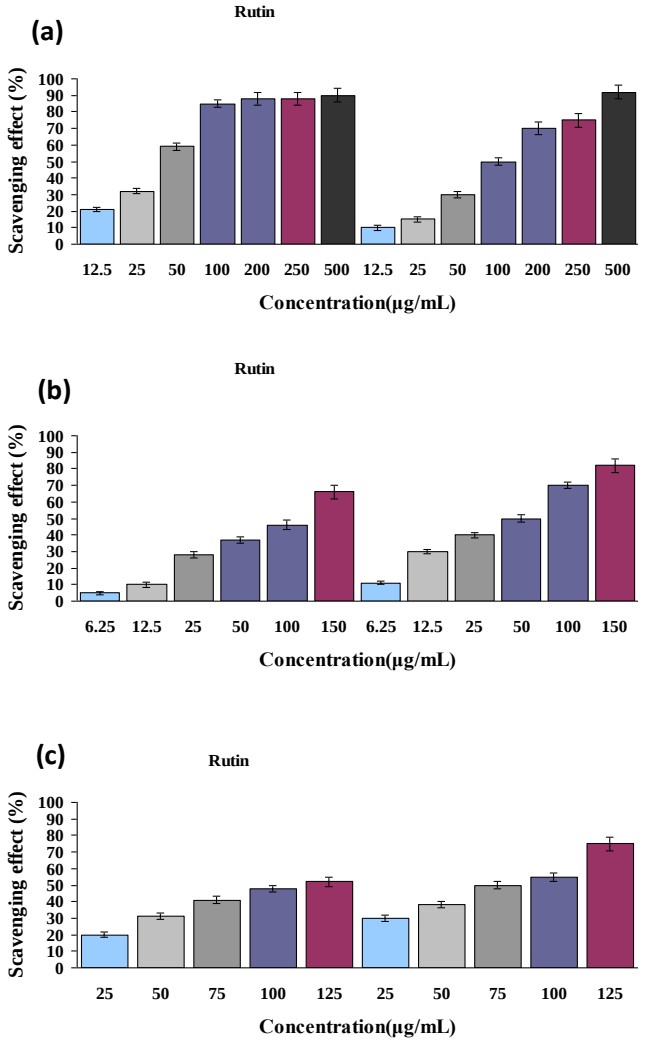

Figure 4. Radical-scavenging activity of total flavonoids from *S. jambos* seeds (TFSJ). Radical-scavenging activities were assessed by measuring the DPPH scavenging activities (a), ABTS radicals scavenging activities (b), and hydroxyl scavenging activities (c).

significant scavenging effects with increasing concentrations in the range of 12.5 to 500 µg/ml.

However, the scavenging effect of TFSJ was significantly lower than that of rutin. At 200 µg/ml, TFSJ and rutin exhibited 69.04 and 88.28% inhibition, respectively, and the EC_{50} values were 95.21 and 40.03 µg/ml for TFSJ and rutin, respectively (Table 4). The different concentrations of TFSJ showed antioxidant activities in a dose dependent manner in the DPPH radical scavenging and the different concentrations of TFSJ also showed antioxidant activities in a dose dependent manner in the DPPH radical scavenging.

Effect of scavenging ABTS radicals

ABTS assay is often used in evaluating total antioxidant power of single compounds and complex mixtures of various plants (Luo et al., 2010). Specific absorbance at 734 nm can be used in both organic and aqueous solvents as an index reflecting the antioxidant activity (Wootton-Beard et al., 2011). In the experiment, the scavenging ability of total flavonoids from *S. jambos* seeds on ABTS free radical was shown in Figure 4B. Their scavenging powers correlated well with increasing concentrations. Aliquots of TFSJ or rutin (100 µg/ml) exhibited 72.65 and 48.33% inhibition, respectively. The EC_{50} value of TFSJ on ABTS radical scavenging activity was found to be 45.79 µg/ml, whereas the EC_{50} value of rutin was found to be 133.41 µg/ml (Table 4). Therefore, when compared to rutin, the ABTS scavenging activity of the extract was high. This could be due to the presence of reactive bioactive constituents and the mixture of other nutrients in the extract.

Scavenging effects on hydroxyl radicals

Hydroxyl radicals are extremely reactive free radicals formed in biological systems and have been implicated as a highly damaging species in free radical pathology, capable of damaging almost every molecule found in living cells (İlhami, 2006). Hydroxyl radicals are very strongly reactive oxygen species, and there is no specific enzyme to defend against them in humans (Liu et al., 2006). Therefore, it is important to discover chemicals with good scavenging capacity for these reactive oxygen species. The hydroxyl radical scavenging capacity of an extract is directly related to its antioxidant activity (Mandade et al., 2011). All results showed antioxidant activity in a dose dependent manner. The EC_{50} values of TFSJ and rutin were found to be 65.22 and 121.78µg/ml, (Table 4C). The ability of the TFSJ extracts to quench hydroxyl radicals seems to be directly related to the prevention of propagation of lipid peroxidation; because TFSJ seems to be a good scavenger of active oxygen species, it will thus reduce the rate of the chain reaction.

Conclusion

Flavonoids are very important constituents of plants because of the scavenging ability conferred by their hydroxyl groups. The flavonoids may contribute directly to anti-oxidative action. In the present study, we demonstrated and optimization TFSJ possesses considerable amounts of flavonoids by response surface methodology (11.4330 mg/g rutin equivalent of extract after optimization). Rutin, a lipid-soluble analogue of flavonoids, was also used as a reference antioxidant compound. The data obtained clearly indicate that the extract possesses potent ABTS scavenging activity and hydroxyl radicals activity, at levels superior to rutin. The TFSJ was able to scavenge DPPH, although less effectively than rutin. These antioxidant activities could

Table 4. Effect of total flavonoids on different radical scavenging activities.

Sample	EC_{50} (μg/ml) [a]		
	DPPH radical scavenging activity	ABTS radical scavenging activity	Hydroxyl radical scavenging activity
TFSJ	95.21±1.78	45.79±1.02	65.22±0.93
Rutin	40.03±2.04	133.41±2.86	121.78±1.74

[a]EC_{50} is a measure of radical scavenging activity being the concentration enquired to inhibit 50% free radical activity.

have contributed, at least partly, to the therapeutic benefits of the certain traditional claims for *S. jambos* seeds. In view of the potential use of *S. jambos* seeds in the functional food industry, its therapeutic benefits and bioactive compounds warrant further investigation.

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