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Optimization of Microwave Assisted Extraction of Total Triterpenoid in *Diospyros kaki* Leaves Using Response Surface Methodology

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The triterpenoid in *Diospyros kaki* leaf has multiple pharmacological activities. Thus a fast and efficient method for preparation of triterpenoid from *Diospyros kaki* is very important. In this study, several extraction methods (Soxhlet extraction, reflux extraction, ultrasound assisted extraction, microwave assisted extraction) were compared. Microwave assisted extraction was found to be the most effective method and then the extraction conditions were further optimized using central composite design. The results showed extraction time and solvent to solid ratio were statistically most significant factors on total triterpenoid extraction. The optimal conditions were calculated as extraction under microwave power 365.3 W for 13.05 min, with 60.27 % ethonal as solvent at a solvent to solid ratio 22.26. Under the optimal conditions, the yield of total triterpenoid obtained experimentally (61.82 mg/g dry leaves) was close to its predicted value of 71.43 mg/g dry leaves, which suggested the mathematical model was fit well with the experimental data of total triterpenoid extraction. In addition, the HPLC assay implied that five triterpene acids (ursolic acid, oleanic acid, barbinervic acid, rotungenic acid and 24-hydroxy ursolic acid) were abundant in the extracts.

Key Words: Total triterpenoid, *Diospyros kaki*, Microwave-assisted extraction, Response surface methodology.

INTRODUCTION

Persimmon (*Diospyros kaki*) belongs to the family Ebenaceae and is thought to have originated from southern mainland China. This plant is now widespread in the tropics and subtropics, including China, Korea and Japan[1,2]. The fruit of persimmon is eaten as fresh or dry fruit and the leaves of this tree are infused with hot (rather than boiling) water and has traditionally been drunk as green tea[3-6] or natural food additive[7,8] for healthcare in Southeast Asia,which can improve the antioxidant activities *in vivo*, inhibit lipid peroxide[9], lower hepatic cholesterol[7] and has become increasingly popular as a health food.

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Since the persimmon leaves have beneficial effects on haemostasis, constipation, hypertension, apoplexy and atherosclerosis, they have also been broadly applied in food and medicinal area[10-16] with few side effects.The leaf of *Diospyros kaki* is an abundant source of amino acids, carotenoids, flavonoids, sugars, tannins, terpenoids and vitamin A. Triterpenoids is the major component and found that triterpenoids isolated from persimmon leaves are mainly responsible for suppressing stimulus-induced superoxide generation and tyrosyl phosphorylation[17], inhibiting protein tyrosine phosphatase 1B (PTP1B) activity[18] and are used as preventives and remedies for type-IV allergy-induced inflammation[14].

Therefore, a fast and efficient method for preparation of triterpenoid from the leaves of *Diospyros kaki* is very important. Many techniques have been developed to extract triterpenoid in some plants, among which the reflux, cooking[19] and solid-liquid extraction[20] are the most commonly used. The traditional methods for the isolation and purification of chemical constituents from plant tissues present some disadvantages. They mainly require longer extraction time, high amounts of solvent and sometimes have lower efficiency. Compared with the traditional methods, microwave assisted extraction (MAE) has many advantages, such as a shorter time, less solvent, higher extraction rate, better production with lower cost[21,22]. The microwaves heat the solvent or solvent mixture directly and the direct interaction of microwaves with the free water molecules presents in the glands and vascular systems, which results in the subsequent rupture of the plant tissue and the release of the active compounds into the organic solvent. Therefore, microwave assisted extraction is an interesting alternative to conventional extraction methods, especially in the case of plant material extraction[23].

In this study, the use of microwave energy to assist extraction of triterpenoid from the leaves of *Diospyros kaki* is presented. In order to assess the superiority of the microwave assisted extraction, several conventional extraction methods were compared. Various experimental factors (irradiation time, microwave power, extractant volume, *etc.*) of microwave assisted extraction on the extraction yield were studied in order to determine the optimal extraction conditions. The purpose of this study is to evaluate microwave assisted extraction with conventional extraction techniques and to develop a novel microwave assisted extraction method for the extraction of triterpenoid from the leaves of *Diospyros kaki*.

EXPERIMENTAL

Diospyros kaki leaves were collected from Fuzhou in Jiangxi Province. All samples were dried and grind to powder with a grinder (TianJin City Taisite Instrument Co., Ltd., China), then passed through different sieves, the 20-40 mesh powder was used for extraction.

Ethanol, petroleum ether (90-120 ºC), acetic acid, acetone, perchloric acid, *n*-butanol, cyclohexane and vanillin are of analytical grade, which were all purchased from local companies. Ursolic acid and oleanic acid were used as standard and supplied by the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Barbinervic acid, rotungenic acid and 24-hydroxy ursolic acid were isolated from the leaves of *Diospyros kaki* previously by the authors' laboratory and their chemical structures were confirmed by EI-MS, ESI-MS, ^{1}H NMR and ^{13}C NMR and HPLC with individual purity not less than 98 %.

HPLC was performed on an Waters2695 liquid chromatography system, equipped with a quaternary solvent delivery system, an autosampler and DAD detector. The column configuration consisted of SunFire C_{18} reserved-phase column (5 μm, 150 mm × 4.6 mm, i.d.). Colorimetric method was performed on a UV762 spectrometer (Shanghai Precision & Scientific Instrument Co., Ltd., Shanghai, China).

Determination of total triterpenoid: Determination of the total content of triterpenoid was performed as described in the literatures[24,25] with a little modification. Briefly, 0.2 mL extract solutions were added to 5 mL tubes, the solvent were heated to evaporation in a water-bath and then 0.3 mL freshly prepared 5 % (w/v) vanillin-acetic acid solution and 1 mL perchloric acid were added. The mixture was shaken to homogeneous and then incubated at 60 ºC for 45 min for complete reaction. Finally, the tubes were taken out and cooled in running water for 2 min, the solution was brought up to 5 mL by adding acetic acid, 5 min later, the absorbance at 547 nm was evaluated using a spectrophotometer. Chromogenic solution contains no extracts was used as reference. Using ursolic acid as standard, the content of triterpenoid can be calculated. The results were expressed as mg triterpenoid/g *Diospyros kaki* leaves.

HPLC assay of triterpenoids: HPLC assay of triterpenoids in the *D. kaki* leaves was performed as described in the literatures[24] with a little modification. In briefly, the ratio 82:18 (v/v) of the solvent system of A-B (A, methanol; B, 0.2 % aqueous H_3PO_4) at a flow rate of 1.0 mL/min was utilized so as to ensure that each run of analysis was completed within 0.5 h with better resolution of adjacent peaks. Column temperature was kept at 25 ºC and 210 nm was chosen as the detection wavelength, as it is close to the maximum absorbency of these triterpene acids.

Extraction method: Dried *D. kaki* leaves powder (20-40 mesh) was pretreated with petroleum ether (90-120 ºC) as described by Fan *et al.*[25] to remove some coloured low polarity impurities so they would not interfere with the detection of total triterpenoid. It will facilitate the subsequent purification steps, through which high purity triterpene acids could be obtained. The defatted powder was stored in a plastic bag and was dried at 80 ºC for 3-5 h before use.

Soxhlet extraction: Defatted powder (1 g) packed with a filter paper was placed in a Soxhlet apparatus and extracted with 100 mL of 80 % aqueous ethanol at the boiling temperature for 2-10 h individually. Afterwards, the solvent was removed from the extract solution in a vacuum evaporator, the extract obtained was weighed and then, the total amount, the recovery and the purity of total triterpenoid in the extracts were determined.

Ultrasound assisted extraction: Ultrasound assisted extraction was performed in an ultrasonic cleaning bath (Kunshan Ultrasonic instruments Co., Ltd., KQ 600DB, China). Working frequency is 40 KHz and the output power is fixed at 600 W. Present preliminary experiments showed that the plastic container is superior to glass vessel in triterpenoid yield aspect (data not shown). This may be due to the reflection of the ultrasonic waves on glass[26]. A 50 mL plastic tube equipped with a reflux condenser was used in ultrasound assisted extraction. Samples (1 g) were placed into this plastic tube and extracted with 25 mL of 60 % (v/v) ethanol. The tube was partially immersed into the bath and its position was fixed in a specific location in ultrasonic field to ensure the *D. kaki* leaves were extracted under the same ultrasound intensity, the water level in the bath was also fixed during the whole process. The experiments were performed for different times at the 60 ºC. After extraction, the tube was removed from the bath and filtered under vacuum when it is still hot and the filtrate was transferred into a volumetric flask for the determination of the total triterpenoid content.

Conventional reflux extraction: For this method, herb matrix (1 g) was extracted with 25 mL of 60 % aqueous ethanol (v/v) in a 100 mL flat-bottomed flask equipped with a condenser, the matrix was stirred at a speed of 500 rpm at 50 ± 1 ºC. After extraction, the extracts were filtered and the total triterpenoid were detected.

Microwave assisted extraction: Microwave assisted extraction was performed in a refitted microwave oven (Hefei Rongshida Sanyo Electric Co. Ltd., 2450 MHz, China). Three holes were drilled on the top of the oven so as to install condenser or agitating device. For microwave assisted extraction, 1 g of sample was extracted in a conical flask which was put on a bracket. After extraction, the extracts were filtered and the concentration was measured by a spectrophotometer.

For the determination of kinetics characteristics of microwave assisted extraction, 25 mL of 70 % aqueous ethanol (v/v) was used, the microwave power was set at 200 W and different extraction times from 2-25 min were examined.

In order to screen the most suitable solvent for microwave assisted extracting, five organic solvents with different polarity (cyclohexane, *n*-butanol, ethanol, ethylacetate, acetone) and three kinds of water solution (pure water, water adjust pH to 3 with acetic acid, water adjust pH to 10 with ammonia) were tested. Experiments were carried out under the following conditions: 10 min microwave irradiation at a microwave power of 233 W, the solvent to solid radio is 10. For further optimizing the extraction conditions, central composite design was performed according to Table-1.

Experimental design: The aim of this study is to establish a rapid and efficient method for extraction of total triterpenoid from the leaves of *Diospyros kaki*. Firstly, four extraction methods involving Soxhlet extraction, reflux extraction, ultrasound assisted extraction and microwave assisted extraction were compared and then preliminary experiments were performed in search of the most effective solvent for microwave assisted extraction, finally, four factors that were expected most influence the process were optimized by response surface methodology.

TABLE-1
CENTRAL COMPOSITE DESIGN ARRANGEMENT WITH THE EXPERIMENTAL AND PREDICTED RESPONSES VALUES FOR YIELD OF TOTAL TRITERPENOID

Run order	Variable levels				Yield of total triterpenoid yields	
	x_1 (X_1)	x_2 (X_2)	x_3 (X_3)	x_4 (X_4)	Actual value	Predicted value
1	-1 (50)	-1 (8)	-1 (15)	-1 (225)	49.39±3.05	50.15
2	1 (70)	-1 (8)	-1 (15)	-1 (225)	55.68±3.14	54.57
3	-1 (50)	1 (16)	-1 (15)	-1 (225)	55.23±2.19	55.60
4	1 (70)	1 (16)	-1 (15)	-1 (225)	55.77±3.22	56.86
5	-1 (50)	-1 (8)	1 (25)	-1 (225)	52.36±1.54	54.63
6	1 (70)	-1 (8)	1 (25)	-1 (225)	56.57±0.60	57.41
7	-1 (50)	1 (16)	1 (25)	-1 (225)	59.92±1.82	60.76
8	1 (70)	1 (16)	1 (25)	-1 (225)	60.14±3.08	60.37
9	-1 (50)	-1 (8)	-1 (15)	1 (475)	51.62±1.72	53.10
10	1 (70)	-1 (8)	-1 (15)	1 (475)	55.96±2.61	56.19
11	-1 (50)	1 (16)	-1 (15)	1 (475)	55.31±4.18	55.54
12	1 (70)	1 (16)	-1 (15)	1 (475)	56.02±2.38	55.46
13	-1 (50)	-1 (8)	1 (25)	1 (475)	58.26±4.02	58.24
14	1 (70)	-1 (8)	1 (25)	1 (475)	58.34±2.38	59.68
15	-1 (50)	1 (16)	1 (25)	1 (475)	58.53±1.84	61.35
16	1 (70)	1 (16)	1 (25)	1 (475)	59.32±1.62	59.63
17	-2 (40)	0 (12)	0 (20)	0 (350)	51.70±2.53	48.72
18	2 (80)	0 (12)	0 (20)	0 (350)	51.21±1.37	51.42
19	0 (60)	-2 (4)	0 (20)	0 (350)	56.74±1.90	55.25
20	0 (60)	2 (20)	0 (20)	0 (350)	61.93±2.51	60.65
21	0 (60)	0 (12)	-2 (10)	0 (350)	53.78±2.10	53.92
22	0 (60)	0 (12)	2 (30)	0 (350)	65.49±4.16	62.57
23	0 (60)	0 (12)	0 (20)	-2 (100)	61.27±1.28	60.01
24	0 (60)	0 (12)	0 (20)	2 (600)	63.73±3.53	62.22
25	0 (60)	0 (12)	0 (20)	0 (350)	68.46±4.19	68.01
26	0 (60)	0 (12)	0 (20)	0 (350)	66.96±5.38	68.01
27	0 (60)	0 (12)	0 (20)	0 (350)	69.51±4.99	68.01
28	0 (60)	0 (12)	0 (20)	0 (350)	65.91±2.81	68.01
29	0 (60)	0 (12)	0 (20)	0 (350)	67.16±2.11	68.01
30	0 (60)	0 (12)	0 (20)	0 (350)	68.86±0.43	68.01
31	0 (60)	0 (12)	0 (20)	0 (350)	69.22±3.51	68.01

The central composite face centered design[27] was chosen for response surface methodology, with a quadratic model and $\alpha = 2$ in this study. Four independent variables, namely ethanol concentration (X_1), extraction time (X_2), solvent to solid radio (X_3) and microwave power (X_4) were studied, with each independent variable varied at five levels represented by $-\alpha$, -1, 0, +1 and $+\alpha$ (Table-1). The variables X_i were coded as x_i according to eqn. 1:

$$x_i = \frac{X_i - \overline{X}_i}{\Delta X_i} \tag{1}$$

where $\overline{X}_i$ is the real value of an independent variable at the center point and ΔX_i the step change value. The design consists of sixteen factorial points, eight axial points and seven center points, leading to 31 sets of experiments (Table-1). All trials were carried out in triplicate and the averages of total triterpenoid yields were taken as responsive value. The experiments were performed randomly to minimize the effects of uncontrolled factors that could have introduced bias into the measurements.

$$Y = b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{i=1}^{n} b_{ii} x_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} b_{ij} x_i x_j \tag{2}$$

The data obtained were then fitted to the following second-order polynomial equation (eqn. 2). Where Y is the yield of total triterpenoid extraction (mg/g), b_0 is the offset term (constant), b_i are the linear coefficients, b_{ii} are the quadratic coefficients, b_{ij} are the interaction coefficients and x_i, x_j are the values of variables. The significance of the second-order model as shown in eqn. 2 was evaluated by analysis of variance (ANOVA). The F (Fisher) test and the final model were obtained. The model was then used to optimize the triterpenoid yield for all the variables studied. Additional experiments were also carried out to verify the predicted optimized conditions.

Statistical analysis: Design Expert software version 7.0.0 (STAT-EASE Inc., Minneapolis, MN) was used to generate the experimental designs, statistical analysis and regression model. The quality of the fit of the polynomial model equation was expressed by the coefficient of determination R^2 and its statistical significance was checked by an F-test.

RESULTS AND DISCUSSION

Comparison of extraction techniques: Fig. 1 describes the mass transfer kinetics of four different extraction methods. The results indicate that Soxhlet extraction is the most inefficient method because it needs a long time (over 8 h) to extract completely, moreover, even its highest extraction yield is still much lower when compared with ultrasound or microwave assisted extraction.

Kinetic curves of ultrasound and reflux extraction can be well compared, because the only difference is that the ultrasound assisted extraction method was conducted under ultrasound field while the reflux extraction was conducted with magnetic stirring. Many authors reported that with the ultrasonic effect of acoustic cavitations, the solid and liquid particles were vibrated and accelerated, as a result the solute quickly diffused from solid phase to the solvent 28-30. Data shown in Fig. 1 verified this point as the extraction yield of ultrasound assisted extraction is much higher than that of reflux extraction.

However, microwave assisted extraction is the most favourable extraction technique because it provides not only high extraction yield but also high efficiency (5 min is enough to give a rather high yield) (Fig. 1). This may be explained by the direct relationship between temperature and solubility and because of the reduction of solvent viscosity with increasing temperature leads to increase in diffusion and increase solvent/solute interaction[31]. Additionally, because microwave assisted extraction allows the sample to be stirred during the heating process, a more homogeneous solution is created and solvent/solute interaction is increased.

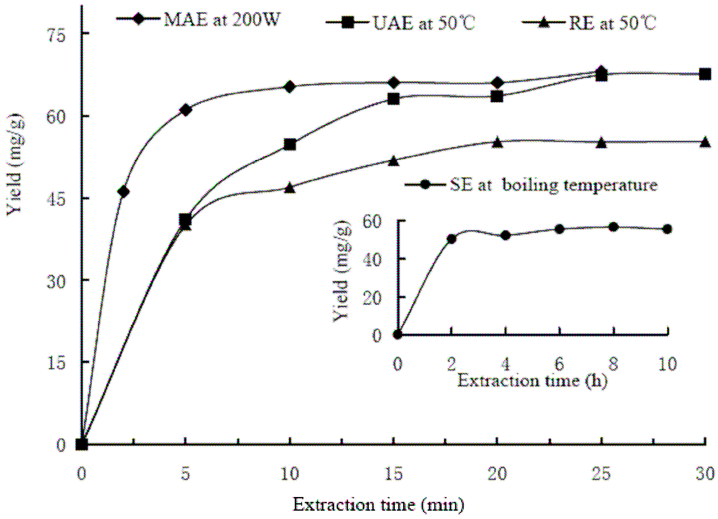

Fig. 1. Kinetics of extraction of Soxhlet extraction (SE), reflux extraction (RE), ultrasound assisted extraction (UAE) RE and microwave assisted extraction (MAE)

Based on the analysis above, the microwave assisted extraction method was chosen for further optimization.

Screening study of microwave assisted extraction solvents: Although the effect of solvent on the extraction efficiency of total triterpenoid from *Diospyros kaki* has been reported previously[25], the optimal extraction solvents for microwave assisted extraction cannot always be reasoned directly from those used in conventional methods[32]. Because the solvent plays a different effect in the two methods. Under conventional extraction conditions,the extractability of different solvents depends mainly on the solubility of the compound in the solvent, the mass transfer kinetics of the product and the strength of the solute/matrix interactions[33], but when the extraction was conducted under microwave irradiation, microwave-absorbing properties of the solvent significantly influence the yield.

The ability of solvent to absorb microwave energy is partly depend on the dissipation factor (tan δ) which is the ratio of the dielectric loss (ε'', a measure of the efficiency of converting microwave energy into heat) to dielectric constant (ε', a measure of the polarizibility of a molecule in an electric field)[32]. The dissipation factor is a measure of the ability of the solvent to absorb microwave energy and dissipate that energy in the form of heat. Thus, solvent that has high dissipation factor would favour to extraction process.

Polar molecules and ionic solutions will absorb microwave energy strongly because they have a permanent dipole moment that will be affected by the microwaves, on the contrary, non-polar solvents such as cyclohexane will not heat up when exposed to microwaves.

The results in Fig. 2 demonstrated that ethanol gave the highest extraction efficiency for microwave assisted extraction, followed by acetone which has the highest dissipation factor among the selected solvents. The non-polar cyclohexane is the most inefficient solvent for microwave assisted extraction. It is interesting to find that the alkaline water gives a rather high yield, it is possibly because that triterpenoid shows acidic character and has high solubility in alkaline solution. On the other hand, it is ionic solution that can be easily heated by microwave. This is an important finding because it demonstrates that avoid using organic solvents was possible for triterpenoid extraction by microwave assisted extraction method. However, acid and alkali should be discreetly used in industrial production to prevent equipment corrosion and environment pollution.

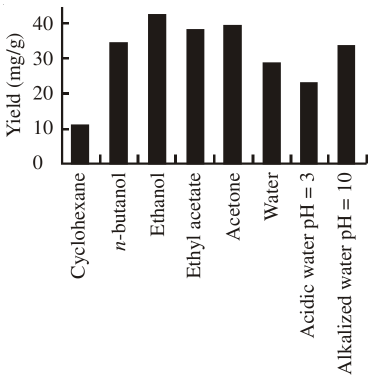

Fig. 2. Effect of extraction solvent on extraction efficiency

From the above finding, ethanol was selected as the most efficient solvent for subsequent investigation. Besides highest yield, it has many advantages such as environmental compatibility, lower toxicity and cost.

Optimization of microwave assisted extraction factors by central composite design: Response surface methodology using central composite design was applied to optimize the levels of significant extraction parameters. Four independent variables, namely ethanol concentration, extraction time, solvent to solid radio and microwave power were studied. The levels of independent variables used in this experiment were determined based on preliminary experiments. Thirty one experiments were carried out from the design and the experimental values were given in Table-1 along with predicted values obtained from the model equation. All the experiments were carried out in triplicate and the mean values of triterpenoid yields were taken for statistical analysis.

Fitting the model: Experimental values of triterpenoid compounds in the extracts were analyzed by multiple regressions to fit the second order regression equation and the regression model in terms of coded factors was predicted in eqn. 3:

$$Y = 68.01 + 0.68x_1 + 1.35x_2 + 2.16x_3 + 0.55x_4 - 0.79x_1x_2 - 0.41x_1x_3 - 0.33x_1x_4 + 0.17x_2x_3 - 0.75x_2x_4 + 0.16x_3x_4 - 4.49x_1^2 - 2.52x_2^2 - 2.44x_3^2 - 1.73x_4^2 \quad (3)$$

The value for the coefficient of determination (R^2) was 0.9454 which implies that over 94 % of the variations for the process efficiency could be explained by the model. The normal plot of residuals (Fig. 3) indicated that the residuals followed a normal distribution. The significance of each coefficient was determined using the F-test and *p*-value. For any of the terms in the models, a large F-value and a small p-value would indicate a more significant effect on the respective response variables[34]. The statistical analysis (Table-2) showed that the model was extremely significant ($p < 0.0001$) and there was no significant lack of fit ($p = 0.1261$), which further validates the model. Based on these statistical tests, the model was accepted to be adequate in representing the actual correlation between the extraction yield and the process variables.

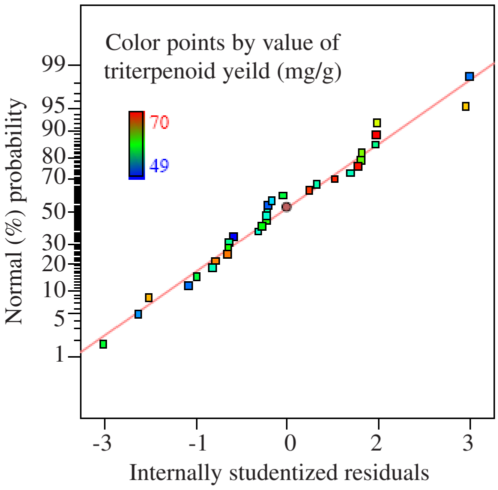

Fig. 3. Normal plot residuals

Among the four parameters, extraction time and solvent to solid radio were found to be statistically most significant factors ($p < 0.05$) on total triterpenoid yield, followed by ethanol concentration and microwave power, which were not significant at 0.05 level. All the linear terms of the four parameters have positive effects while all of their quadratic terms have significant negative effects, which indicating that total triterpenoid yields increased as the level of these factors increased and decreased as the level of these parameters increased above certain values. Six interaction terms exhibited no significant effect.

TABLE-2
ANALYSIS OF VARIANCE (ANOVA) FOR THE SECOND-ORDER RESPONSE SURFACE MODEL

Term	Degree of freedom	Mean square	F-Value	Prob > F
Model	14	72.10	19.80	< 0.0001
x_1	1	10.95	3.01	0.1022
x_2	1	43.86	12.05	0.0032
x_3	1	112.13	30.80	<0.0001
x_4	1	7.30	2.01	0.1759
$x_1 x_2$	1	10.01	2.75	0.1168
$x_1 x_3$	1	2.72	0.75	0.4005
$x_1 x_4$	1	1.78	0.49	0.4942
$x_2 x_3$	1	0.45	0.12	0.7293
$x_2 x_4$	1	9.08	2.50	0.1338
$x_3 x_4$	1	0.43	0.12	0.7350
x_1^2	1	575.49	158.06	<0.0001
x_2^2	1	181.06	49.73	<0.0001
x_3^2	1	170.48	46.83	<0.0001
x_4^2	1	85.12	23.38	0.0002
Lack of fit	10	4.74	2.61	0.1261
Error	6	1.81	–	–
Total error	30	–	–	–

$R^2 = 0.9454$; Adj $R^2 = 0.8977$.

Analysis of response surfaces: Three-dimensional response surfaces and contour plots for the responses are the graphical representations of the regression equations. Response surface graphs were plotted between two independent variables while remaining independent variables were kept at the zero coded level.

Fig. 4 shows how four pairs of extraction parameters effect on the extraction of total triterpenoid from *D. kaki* leaves. All the four surfaces are upper convex, with a maximum point in the center of the experimental domain, this demonstrated that the ranges of factors were chosen properly.

Variation of extraction time and ethanol concentration revealed that total triterpenoid recovery was maximum when time and concentration were 13 min and 60 %, respectively (Fig. 4a). It is quite evident that the yield tend to peak with increased extraction duration at moderate ethanol concentration, although, further duration increments cause decline in triterpenoid yield. This might be due to increased dissolution of the polymer impurities at longer extraction times, causing an increase in viscosity[32], which makes the target analyte absorbed on the sample matrix. From Figs. 4a,c,d it can be concluded that the most suitable ethanol concentration is around 60 %, this result is close to the microwave assisted extraction of antihepatotoxic triterpenoid from *Actinidia deliciosa* root[35], whose optimum solvent is 72.67 % ethanol. Using too much volume of solvent resulted in not only wasting resources but also a decrease of triterpenoid yield (Fig. 4c). Generally, in conventional extraction techniques a higher volume of solvent will increase the recovery, but in microwave assisted extraction a higher solvent volume may give lower recoveries. The similar phenomenon was also reported previously when extract effective constituents from natural plant[36,37]. The possible reason for the decreased efficiency with more solvent is that microwave energy will decay when penetrating too much solvent that the effect on the plant tissue becomes week[38], so microwave assisted extraction does not need much volume of solvent; this is also the point where microwave assisted extraction is superior to conventional extraction. The effect of power was found to be relatively minor, which could be verified from the relatively straight contour line (Fig. 4d).

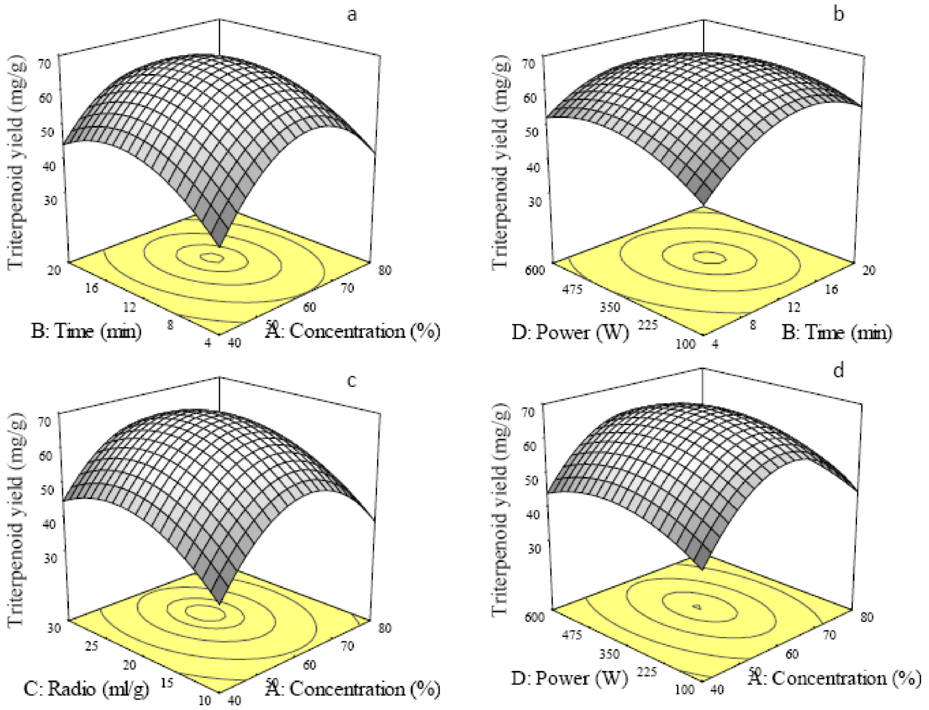

Fig. 4. 3D surface and contour plot showing effect of (a) extraction time and ethanol concentration; (b) Microwave power and extraction time; (c) solvent to solid radio and ethanol concentration; (d) microwave power and ethanol concentration on total triterpenoid extraction

Optimum conditions and model verification: Based on the quadratic model, the optimum conditions for triterpenoid extraction were calculated as follows: ethanol concentration 60.27 %, extraction time 13.05 min, solvent to solid radio 22.26 and microwave power 365.3 W. At these optimum levels of extraction parameters, the average total triterpenoid yield of four repeated experiments was 71.43 mg/g, which is very close to the predicted value of 68.72 mg/g. This demonstrated the model was adequate for reflecting the expected optimization.

Composition of the total triterpenoid in the extracts of *Diospyros kaki* leaf: The compositions of the total triterpenoid in the extracts of *Diospyros kaki* were detected by HPLC assay. The HPLC assay (Fig. 5) implied that five triterpene acids (*i.e.*, ursolic acid, oleanic acid, barbinervic acid, rotungenic acid and 24-hydroxy ursolic acid) were abundant in the *Diospyros kaki* leaf extracts.

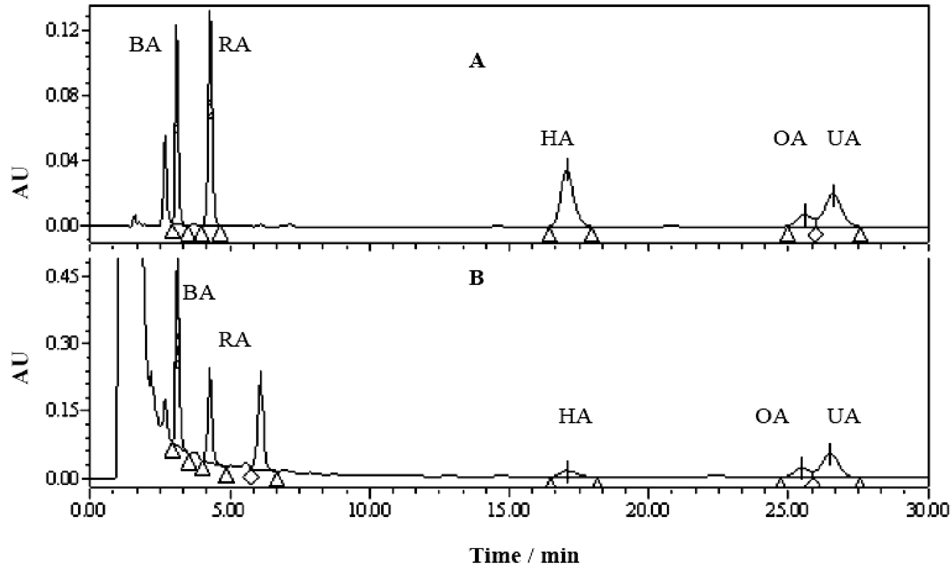

Fig. 5. HPLC chromatograms of five triterpene acid standards (UA = ursolic acid, OA = oleanic acid, BA = barbinervic acid, RA = rotungenic acid, HA = 24-hydroxy ursolic acid) (A) and the extracts from the leaves of *Diospyros kaki* (B)

Conclusion

The present study established an efficient method for extraction of total triterpenoid from the leaves of *Diospyros kaki*. After comparing four different extraction methods (Soxhlet extraction, reflux extraction, ultrasound assisted, extraction, microwave assisted extraction). The microwave assisted extraction was found to be the most efficient technique for higher extraction yield and much shorter time expenditure. A series of extraction solvents with different polar were studied for microwave assisted extraction and ethanol was selected as the most efficient solvent, because it yields the highest amount of total triterpenoid and also it has lower toxicity and cost. By applying central composite design, maximized triterpenoid yield (71.43 mg/g) from *Diospyros kaki* was achieved under the optimal condition. The optimal operation conditions were extraction under microwave power 365.3 W for 13.05 min, with 60.27 % ethonal as solvent at a solvent to solid ratio 22.26 (volume per mass). The microwave assisted extraction proved to be a powerful method, contributing to the development of a more effective technique to extract and recover total triterpenoid from *Diospyros kaki* leaves. In addition, five triterpene acids (ursolic acid, oleanic acid, barbinervic acid, rotungenic acid and 24-hydroxy ursolic acid) were observed abundant in the extracts of total triterpenoid from *Diospyros kaki* leaves by HPLC assay.

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