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Original Research Article

Optimization of extraction of total flavonoids from *Drynaria* rhizome, and its effect on osteoclast differentiation

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Abstract

Purpose: *To optimize the extraction parameters for total flavonoids of Drynaria rhizome, and determine their effect on osteoclast differentiation and bone resorption in vitro.*
Methods: *A 5-level 3-factor central composite design was applied to the optimization of extract yield of total flavonoids from Drynaria rhizome. Response Surface Methodology (RSM) design was used to optimize total flavonoids extraction from Drynaria rhizome. The independent factors included extraction temperature (A), extraction time (B) and liquid: solid ratio (C). Moreover, NFATc1, DC-STAMP, cathepsin K and MMP-9 mRNA expressions were determined.*
Results: *Values obtained were fitted into a second-order polynomial equation with multiple regression analysis using a statistical method. Analysis of variance results indicate that the independent variables influenced total flavonoid extraction from Drynaria rhizome. The optimal conditions for extraction yield were extraction temperature of 75 ^{o}C, extraction time of 100 min, and liquid: solid ratio of 107:1. The yield of 5.38 ± 0.62 % was consistent with these optimized conditions, which was an indication of the accuracy of the model. Experiments revealed that total flavonoids from Drynaria rhizome regulated the expression levels of NFATc1, DC-STAMP, cathepsin K and MMP-9 mRNA*
Conclusion: *This study has successfully optimized the extraction yield of total flavonoids from Drynaria rhizome. The total flavonoids inhibit osteoclast differentiation and bone resorption. Thus, they may be beneficial in the treatment of bone diseases.*

Keywords: *Flavonoids, Drynaria rhizome, RSM, Osteoclast differentiation, Bone resorption*

INTRODUCTION

Drynaria fortunei (Kunze) J.Sm. (Polypodiaceae) is a fern that grows in South China. In Traditional Chinese Medicine (TCM), the rhizomes of this fern are used for treating broken bones and osteoporosis [1,2]. Studies have demonstrated that *Drynaria fortunei* contains flavonoids, triterpenes and phenylpropanoids [3,4].

In *in vitro* experiments on osteoblastic cells, *Drynaria fortune* has showed anti-resorptive properties [5]. Moreover, it has been reported that oral administration of *Drynaria fortunei* extracts to mice enhanced bone mineral density [6]. In traditional system of medicine, *Drynaria fortune* rhizome has been employed for treating hyperlipidemia, inflammation, rheumatism, and arteriosclerosis, as well as skin diseases such as leprosy, malignant furuncle, and gangrene [7-10].

Response surface methodology (RSM) is a procedure in statistics and mathematics which is employed for optimizing multifactorial processes, and for expressing the effect of multiple factors on the response. Response surface methodology is an effective and reliable method for the prediction of optimum conditions for maximization or minimization of different independent factors, and the association between responses and independent variables. In this process, factors that make significant impacts on prediction can be identified through the use of parameter estimate [11]. Response surface methodology (RSM) is applied for process optimization in various aspects of food formulations [12].

The aim of the current was to optimize the individual and interactive effects of extraction temperature, extraction duration and liquid: solid ratio on the yield of flavonoid extract from *Drynaria* rhizome by RSM, and to investigate the effect of the extract on differentiation of osteoclasts in RANKL-treated RAW 264.7 cell line.

EXPERIMENTAL

Material and methods

Drynaria rhizome was bought from Merro Pharmaceutical Co. Ltd (DaLian, China) and identified by expert Xu.

Reflux extraction

Drynaria rhizome (50 g) was extracted with 70 % ethanol solution at different temperatures and for different durations. Each extract was evaporated to a volume of 150 mL at low pressure at 50 ^oC. The effect of temperature, extraction time and liquid: solid ratio as tested variable parameters were evaluated by examining extraction yield.

Experimental design

A three-factor and five-level central composite design (CCD) with RSM was used to ascertain how the independent variables could be optimally combined. The variables investigated were extraction time, extraction temperature, and liquid: solid ratio. Flavonoid yield was considered as the dependent variable. These factors were selected on the basis of prior data from single-factor studies. In the experimental design, twenty runs were needed for a CCD of 3 independent variables at 3 levels. The design of experiments is shown in Table 1.

Cell culture

RAW 264.7 cell line was got from American Type Culture Collection (ATCC, Rockville, USA). The cells were cultured in high glucose DMEM containing 10 % FBS and 1 % penicillin/streptomycin.

Osteoclast differentiation

The cells were seeded at a density of 5 × 10^3 cells/well in 24-well plates. After 5 days, they were divided into five groups: control, RANKL (50 ng/mL), RANKL (50 ng/ml) + total flavonoids extract of *Drynaria* rhizome (10 mg/L), RANKL (50 ng/mL) + total flavonoid extract of *Drynaria* rhizome (20 mg/L), and RANKL (50 ng/mL) + total flavonoid extract of *Drynaria* rhizome (30 mg/L). The cultured cells were subjected to fixation and stained with TRAP. Osteoclasts were recognized as cells that were positively stained by TRAP, with the presence of at least three nuclei.

Real-time polymerase chain reaction (PCR) analysis

Table 1: Genes used and their sequences

Gene	Sequence
NFATc1	Forward, 5′-GGAGAGTCCGAGAATCGAGAT-3′; Reverse, 5′-TTGCAGCTAGGAAGTACGTCT-3′
DC-STAMP:	Forward, 5′-TCCTCCATGAACAAACAGTTCCA-3′; Reverse, 5′-AGACGTGGTTTAGGAATGCAGCTC-3′
Cathepsin K:	Forward, 5′-ACGAGAAAGCCCTGAAGA-3′; Reverse, 5′-TGTAAAACTGGAAAGATGCC-3′
MMP-9:	Forward, 5′-TGAATAAAGACGACATAGACGGCAT-3′; Reverse, 5′-GGTAGTGGGACACATAGTGGGAG-3′

The extraction of total cellular RNA was carried out using Trizol reagent. The extracted RNA was converted to cDNA through reverse transcription with appropriate reverse transcription kit (Toyobo ReverTra Ace qPCR RT Master Mix, Japan). The RT-PCR was performed using SYBR® Green RT-PCR Master Mix (Toyobo, Japan), and reaction products were determined using StepOnePlus Real-time PCR system (Thermo Fisher, USA). RT-PCR was carried out under the following steps and conditions: 95 ^oC for 1 min, 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 45 s, followed by melting curve analysis. The primer sequences used are shown in Table 1.

Statistical analysis

The results are shown as mean ± SEM. Statistical analysis was performed using ANOVA followed by Tukey's post-test. Comparison of the two groups was carried out with Student's *t*-test. GraphPad Prism software was employed for the statistical analysis. Values of $p < 0.05$ were assumed to indicate statistical significance of differences.

RESULTS

Effect of variables on extraction yield

Extraction temperature, extraction duration and liquid: solid ratio were selected as independent variable parameters. Figure 1 reveals that extraction yield was enhanced by temperature increases to a certain extent. When extraction temperature exceeded 80 °C, extract yield was 5.42 %, but it did not change subsequently.

Figure 2 reveals that extraction yield increased with increase in extraction time up to some extent. When extraction time exceeded 105 min, extraction yield was 5.49 %, but it did not change subsequently.

Figure 3 shows that extraction yield increased initially with increases in liquid: solid ratio. However, when the liquid: solid ratio exceeded 110:1, extraction yield was 5.31 %. No further changes were seen beyond this point.

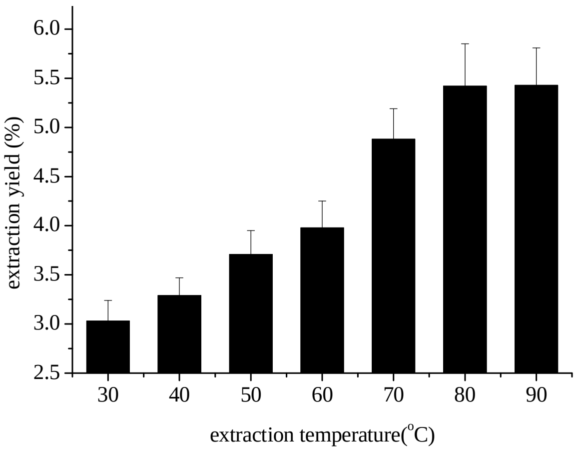

Figure 1: Effect of extraction temperature on yield of total flavonoids from *Drynaria* rhizome

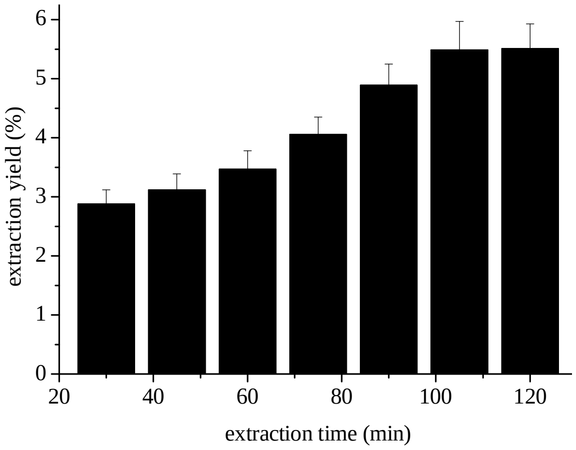

Figure 2: Effect of extraction time on yield of total flavonoids from *Drynaria* rhizome

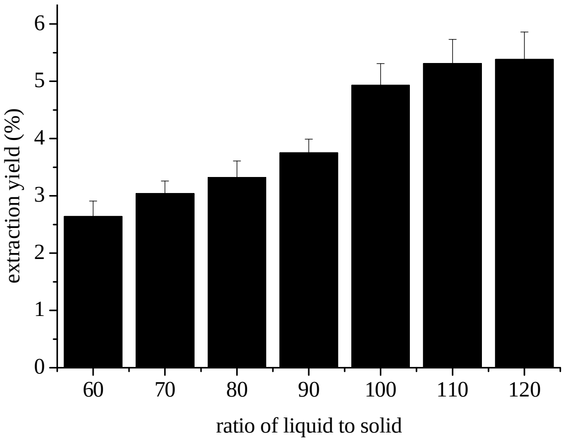

Figure 3: Effect of liquid: solid ratio on yield of total flavonoids from *Drynaria* rhizome

Optimization of factors using central composite design

The central composite design and extraction yields are shown in Table 2. Design-Expert software was used for the development of response surface models. The results of ANOVA in respect of response surface quadratic model are shown in Table 3. This model was highly significant, based on F- and low probability values of 15.84 and $p < 0.0001$, respectively. The model terms A, C, AC, A^2 and C^2 were significant, and AC implied a marked association between extraction temperature and liquid: solid ratio. The high R^2 (0.9345) meant a significant agreement between the predicted and experimental values. Equation 1 shows the quadratic model for predicting the extraction yield.

*R = +5.19 + 0.34*A + 1.498E - 003*B + 0.35*C + 0.049*A*B - 0.28*A*C - 0.046*B*C - 0.13*A2 - 0.014*B2 - 0.13*C2 ………. (1)*

Table 2: Box-Behnken design (BBD) showing A, B and C responses to changes in conditions of extraction

Run	A: Extraction temperature (°C)	B: Extraction time (min)	C: Ratio of liquid to solid	R: Extraction yield (%)
1	-1.00	-1.00	-1.00	3.75
2	1.00	-1.00	-1.00	5.02
3	-1.00	1.00	-1.00	3.72
4	1.00 (80)	1.00	-1.00 (90)	5.22
5	-1.00 (60)	-1.00	1.00	5.24
6	1.00	-1.00 (75)	1.00	5.44
7	-1.00	1.00	1.00	5.06
8	1.00	1.00 (105)	1.00 (110)	5.42
9	-1.68 (53.2)	0.00 (90)	0.00 (100)	4.5
10	1.68 (86.8)	0.00	0.00	5.31
11	0.00 (70)	-1.68 (64.8)	0.00	5.22
12	0.00	1.68 (115.2)	0.00	5.25
13	0.00	0.00	-1.68 (83.2)	4.52
14	0.00	0.00	1.68 (116.8)	5.3
15	0.00	0.00	0.00	5.2
16	0.00	0.00	0.00	5.19
17	0.00	0.00	0.00	5.18
18	0.00	0.00	0.00	5.16
19	0.00	0.00	0.00	5.18
20	0.00	0.00	0.00	5.21

Table 3: Regression coefficient (β), coefficient of determination (R^2) and F-test values of the predicted second order polynomial models (BBD) for total flavonoid extraction from *Drynaria* rhizome

Source	Sum of squares	df	Mean square	F	*P* Prob > F	
Model	4.36	9	0.48	15.84	< 0.0001	Significant
A-Extraction temp. °C	1.61	1	1.61	52.7	< 0.0001	
B-Extraction time (min)	3.06E-05	1	3.06E-05	1.00E-03	0.9754	
C-liquid: solid	1.66	1	1.66	54.27	< 0.0001	
AB	0.019	1	0.019	0.62	0.4488	
AC	0.61	1	0.61	19.96	0.0012	
BC	0.017	1	0.017	0.56	0.4717	
A^2	0.25	1	0.25	8.05	0.0176	
B^2	2.85E-03	1	2.85E-03	0.093	0.7665	
C^2	0.24	1	0.24	7.83	0.0188	
SD			0.17	R^2		0.9345
Mean			5	Adjusted R^2		0.8755
Coeff. var (%)			3.5	Predicted R^2		0.5047
Precision			2.31	Precision adequacy		11.844

Figure 4 (I) shows the 3-dimensional surface plots between each pair of independent variables when the 3rd variable was fixed. These plots reveal the most critical variables in total flavonoids extraction from *Drynaria* rhizome were liquid: solid ratio and temperature. When liquid: solid was constant, extract yield was rapidly enhanced as temperature was raised. In contrast, the duration of extraction did not have much impact on the yield of total flavonoids from *Drynaria* rhizome.

Figure 4 (II) shows 3-dimensional graphic surface and contour plots of combined effects of temperature and liquid: solid ratio on yield level of total flavonoids. The plots were generated from two-variable responses at fixed middle status of the 3rd variable. A strong association was found between the two variables, as revealed from the contour and shape of the plots. The yield of total flavonoids was high in the temperature range of 60 - 80 °C, and liquid: solid ratio in the range of 90:1 - 110:1. This demonstrates that these two variables had significant influence on the yield of total flavonoids, which is consistent with the findings shown in Table 2.

Figure 4(III) shows the influence of duration of extraction and liquid: solid ratio on total flavonoid yield (Y). The yield was rapidly enhanced by increasing liquid: solid ratio, but increased at a slow rate in response to increasing duration of extraction.

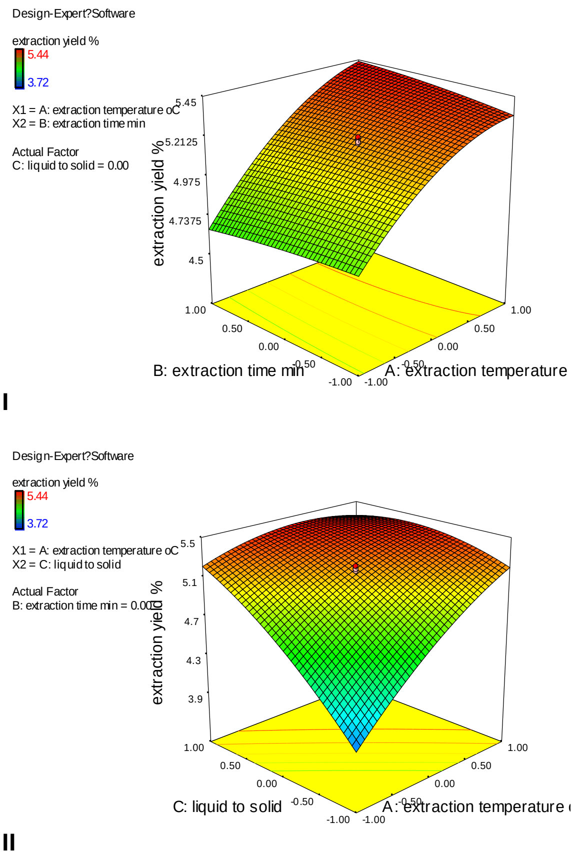

Figure 4: Response surface and contour plots showing the interaction effects of (I) A and B, (II) A and C on the extraction yield of total flavonoids from *Drynaria* rhizome. A: extraction temperature; B: extraction time; C:liquid: solid ratio

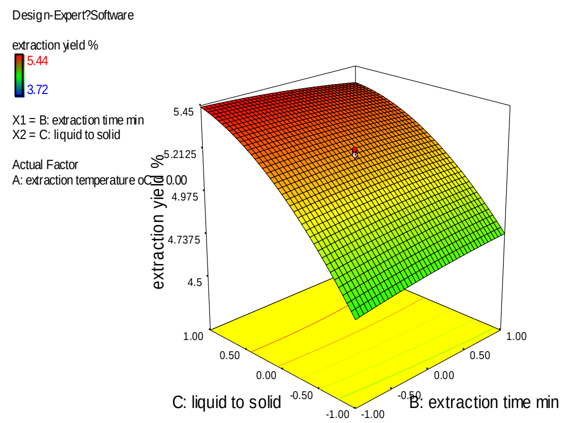

Figure 5: Response surface and contour graphs depicting the influence of interaction of B and C on total flavonoid yield from *Drynaria* rhizome. B: extraction time; C: liquid: solid ratio

Model validation

The Design-Expert 9 predicted total flavonoid yield of 5.46 %, which was very close to actual mean extract yield of 5.38 ± 0.62 % under the software-suggested optimum conditions. The closeness of the predicted and actual yields point to the reliability of the parameter optimization. The optimal conditions for determining extraction yield were extraction temperature of 75 ^{o}C, extraction time of 100 min, and liquid: solid ratio of 107:1.

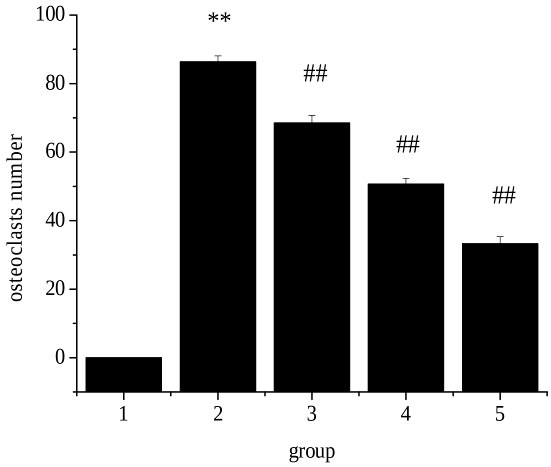

Figure 6: Effect of total flavonoids from yield rhizome on osteoclasts number. 1: control; 2: RANKL (50 ng/mL); 3: RANKL (50 ng/mL) + total flavonoid extract of *Drynaria* rhizome (10 mg/L); 4: RANKL (50 ng/mL) + total flavonoid extract of *Drynaria* rhizome (20 mg/L); 5: RANKL (50 ng/mL) + total flavonoid extract of *Drynaria* rhizome (30 mg/L)

Effect of total flavonoids of *Drynaria* rhizome on osteoclast differentiation in RANKL-stimulated RAW 264.7

RAW 264.7 cells were treated with RANKL and various concentrations of total flavonoid extract of *Drynaria* rhizome (10, 20 and 30 mg/L). As shown in Figure 6, total flavonoid extract of *Drynaria* rhizome (10, 20 and 30 mg/L) dose-dependently inhibited the differentiation of osteoclast.

Effect of total flavonoids of *Drynaria* rhizome on osteoclast differentiation-related gene expressions in RANKL-stimulated RAW 264.7

As shown in Figure 7, NFATc1, DC-STAMP, cathepsin K and MMP-9 mRNA expression levels were markedly downregulated, when compared with corresponding values in control group. Treatment with total flavonoid extract of *Drynaria* rhizome (10, 20 and 30 mg/L) dose-dependently increased NFATc1, DC-STAMP, cathepsin K and MMP-9 mRNA expression levels.

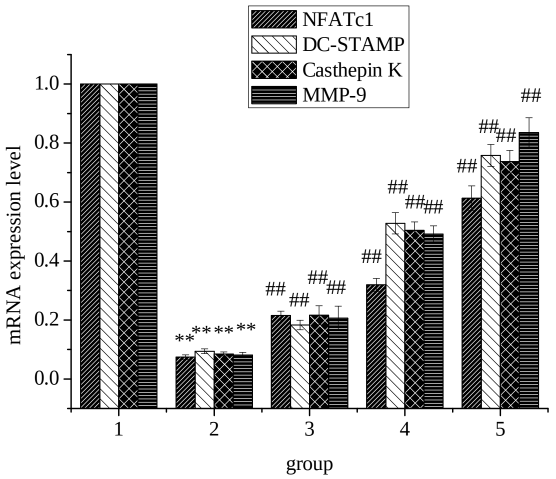

Figure 7: Effect of total flavonoids from *Drynaria* rhizome on NFATc1, DC-STAMP, cathepsin K and MMP-9 mRNA expression levels. 1: control; 2: RANKL (50 ng/mL); 3: RANKL (50 ng/mL) + total flavonoid extract of *Drynaria* rhizome (10 mg/L); 4: RANKL (50 ng/mL) + total flavonoid extract of *Drynaria* rhizome (20 mg/L); 5: RANKL (50 ng/mL) + total flavonoid extract of *Drynaria* rhizome (30 mg/L)

DISCUSSION

The closeness of the predicted and actual yields of total flavonoids point to the reliability of the parameter optimization. Osteoporosis is one of the diseases treated with the use of TCM [13]. Indeed, *Drynaria fortunei* has been employed for centuries as therapy for osteoporosis [14]. Studies on ovariectomized rat model have shown that osteopractic total flavone enhanced their bone mineral density and bone histomorphometry [15]. Osteoclasts are important in bone resorption and skeletal development [16]. Moreover, osteoclasts control the differentiation of osteoblasts, enhance the mobilization of hematopoietic stem cells from bone marrow to the bloods, and take part in immune response [17].

The results of the present study showed that total flavonoids extracted from *Drynaria* rhizome dose-dependently decreased RANKL-induced TRAP-positive osteoclast number. Results from RT-PCR revealed that the total flavonoid extract of *Drynaria* rhizome may affect the mRNA expressions of osteoclast differentiation- related genes NFATc1 and DC-STAMP mRNA. In addition, RT-PCR results indicated that the total flavonoid extract may affect the mRNA expression levels of osteoclast bone absorption activity-related genes cathepsin K and MMP-9. These results suggest that total flavonoids from *Drynaria* rhizome may decrease osteoclasts bone resorption activity.

CONCLUSION

The optimization of parameters for total flavonoid extraction from *Drynaria* rhizome in the present work is reliable. This study has demonstrated that total flavonoid extract of *Drynaria* rhizome dose-dependently regulate RANKL-induced TRAP-positive osteoclast number. In addition, total flavonoids from *Drynaria* rhizome may change mRNA expression levels of osteoclast differentiation-related genes NFATc1 and DC-STAMP and mRNA expression levels of osteoclast bone resorption activity-related genes cathepsin K and MMP-9 mRNA. These results indicate that total flavonoid extract of *Drynaria* rhizome inhibits osteoclast differentiation and bone resorption. This finding is beneficial to bone regeneration and merits clinical application.

DECLARATIONS

Acknowledgement

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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