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Response surface methodology to extraction of dioscoreae polysaccharides and the effects on rat's bone quality

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ABSTRACT

The objective of this study was to determine the optimum extraction conditions for dioscoreae polysaccharides. The optimum conditions were evaluated with fractional factorial design and optimized using response surface methodology. The effects of three independent variables, namely extraction time, extraction temperature and ratio of liquid to solid on the yield of dioscoreae polysaccharides were investigated. Results indicated that the data were adequately fitted into three second-order polynomial models. The independent variables, the linearity of extraction time and temperature, the quadratics of extraction temperature and ratio of liquid to solid had a significant effect on the yield of polysaccharides. The optimal extraction parameters were extraction time of 140 min, temperature of 100 °C and the ratio of liquid to solid of 3, according to the response surface analysis. Pharmacology experiment showed that dioscoreae polysaccharides decreased serum ALP, IL-6 and BGP levels, and enhanced BMD, BMC, bone Hyp, Ca^{2+} and P^{3+} levels.

1. Introduction

Osteoporosis, a common disease characterized by a decreased bone mineral density (BMD) and a microarchitectural deterioration of bone structure, is a chronic condition chiefly affecting postmenopausal women, in whom the skeleton loses a significant percentage of its mineralized mass and mechanical resiliency, thereby becoming prone to fracture (Deyhim et al., 2006). Plenty of studies around this disease have been carried out in recent years. The etiology of human osteoporosis is multifactorial, resulting from an interplay of genetic components, hormonal excess or deficiency, dietary factors, and physical activity (Hulley, Conradie, Langeveldt, & Hough, 2002; Okumura et al., 1987). As the population ages, the incidence of hip fractures and costs for treatment will rise dramatically in the future, unless effective prophylactic measures are taken (Nagareddy & Lakshmana, 2005).

Nature medicines have been investigated for some wonderful pharmacological activities (Alim et al., 2009; Yongabi, Harris, Lewis, & Agho, 2009; Yongabi, Mbacham, Nubia, & Singh, 2009; Kim et al., 2009; Rezazadeh et al., 2009). Chinese traditional medicine, Rhizoma dioscoreae, is the root of Dioscorea opposita Thunb (Zhang, Liu, & Chen, 2004). It is useful for treating the angiocardiopathy, controlling blood pressure and improving immunity ability and also can be used as an anti-decrepitude tonic (McAnuff, Omoruyi, Morrison, & Asemota, 2002; McAnuff-Harding, Omoruyi, & Asemota, 2006). In China, dioscoreae is also used as tonic nourishment and is considered a potential functional food. Effects of dioscoreae on bone quality have not been investigated.

Response surface methodology (RSM) is a mathematical tool, which can help in arriving at optimum conditions for a reaction with minimum number of experiments to obtain statistically acceptable results. RSM enables evaluation of the effects of multiple parameters, alone or in combination, on response variables and also predicts their behavior under given sets of conditions (Chiang, Shih, & Chu, 2001). The objective of this study was to optimize the extraction time, extraction temperature, and ratio of liquid to solid by RSM for maximum yield of dioscoreae polysaccharides. Then, effects of dioscoreae on bone quality in rats were investigated.

2. Materials and method

2.1. Preparation of the dioscoreae polysaccharides

Dioscoreae was collected in November 2004 from a local herb market and stored at room temperature in a dry place prior to use. The root parts of dioscoreae were dried at ambient temperature. Then, distilled water was added to 100 g of dried dioscoreae finely powered and the mixture was heated under reflux for certain

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Table 1
Factors and levels.

Factor	Low	Center	High
X_1	−1 (80 min)	0 (110 min)	1 (140 min)
X_2	−1 (80 °C)	0 (90 °C)	1 (100 °C)
X_3	−1 (2)	0 (3)	1 (4)

time and then the decoction was filtered. The filtrate was frozen at −20 °C and lyophilized. The crude yield of the lyophilized material was approximately 18% (w/w), it was stored at ambient temperature until further use.

2.2. Experimental designs

Response surface methodology (RSM) with central composite design (CCD) was employed to investigate the optimal parameters of dioscoreae polysaccharides. Three independent parameters namely, extracting time, extracting temperature and ratio of liquid to solid at three different levels each, were employed. The parameters were chosen and their levels were based on preliminary experiments carried out in our laboratory.

Table 1 gives the range of variables employed. The actual set of experiments performed (experimental runs 1–20) and the yield of dioscoreae polysaccharides obtained are shown in Table 2. A second-order polynomial equation was developed to study the effects of variables on the yield. The equation indicates the effect of variables in terms of linear, quadratic, and cross-product terms:

$$Y = \beta_0 \pm \sum \beta_i X_i \pm \sum \beta_{ii} X_i^2 \pm \sum \beta_{ij} X_i X_j \quad (1)$$

where Y is the yield of dioscoreae polysaccharides (%), X_i and X_j are the levels of variables, β_0 the constant term, β_i the coefficient of the linear terms, β_{ii} the coefficient of the quadratic terms, and β_{ij} the coefficient of the cross-product terms. All the experimental data were statistically analyzed by the SAS statistical package (version 8.1, SAS Institute, Cary, NC, USA). The graphical representations of the above equation in the form of surface plots were used to describe the individual and cumulative effects of the test variables on the response.

Table 2
Experimental design and responses of the dependent variables to the extract parameters.

Run	X_1	X_2	X_3	Y_1
1	−1.00000	−1.00000	−1.00000	0.9
2	−1.00000	−1.00000	1.00000	1.0
3	−1.00000	1.00000	−1.00000	1.2
4	−1.00000	1.00000	1.00000	1.3
5	1.00000	−1.00000	−1.00000	1.2
6	1.00000	−1.00000	1.00000	1.4
7	1.00000	1.00000	−1.00000	1.5
8	1.00000	1.00000	1.00000	1.4
9	−1.68179	0.00000	0.00000	1.2
10	1.68179	0.00000	0.00000	1.5
11	0.00000	−1.68179	0.00000	1.2
12	0.00000	1.68179	0.00000	1.4
13	0.00000	0.00000	−1.68179	1.2
14	0.00000	0.00000	1.68179	1.3
15	0.00000	0.00000	0.00000	1.4
16	0.00000	0.00000	0.00000	1.4
17	0.00000	0.00000	0.00000	1.4
18	0.00000	0.00000	0.00000	1.4
19	0.00000	0.00000	0.00000	1.4
20	0.00000	0.00000	0.00000	1.4

2.3. Fourier transform infrared spectroscopy (FT-IR)

FT-IR spectra of polysaccharides KBr pellet were obtained with a FT-IR spectrometer (Spectrum One Version B, Perkin Elmer, America) (Huang, Wu, Wei, Liao, & Chen, 2010).

2.4. Animal group and treatment

Thirty-two adult male Wister rats, weighing about 170 g, were bred in the animal research center of our university, China. Animals were handled in accordance with laboratory animal welfare guidelines. Animals were housed in 4 groups of 8 animals in a controlled environment of 24 °C and 12 h light (dark cycle from 7 pm to 7 am) with food and water freely available and randomly assigned to the normal control group (1), model control (2), and two polysaccharides-treatment groups (3 and 4). The acclimatized rats were bilaterally ovariectomized using the dorsal approach. The rats were anaesthetized with ether. A single longitudinal skin incision was made on the dorsal midline at the level of the kidneys. Both ovaries were ligated and removed in the model control and two polysaccharides-treatment groups. Success of ovariectomy was confirmed at necropsy by failure to detect ovarian tissue and by observation of marked atrophy of uterine horns. During the surgery, aseptic techniques were used to avoid the potential infection of pathogens.

After the operation, 8 plastic cages (400 mm × 240 mm × 240 mm) were used and 4 rats from the same group were housed in each cage. Each cage was held up by a plastic support with four legs of which height could be freely adjusted.

The rats of the groups 1 (normal control) and 2 (model control) were treated with vehicle alone for 7 weeks.

The rats of the group 3 received dioscoreae polysaccharides by gavage at a dose of 100 mg/kg bw for 7 weeks.

The rats of the group 4 received dioscoreae polysaccharides by gavage at a dose of 200 mg/kg bw for 7 weeks.

At the end of experimental period, rat body weights were recorded. The rats were fasted for 16 h. Animals were subjected to light ether anaesthesia and killed by cervical dislocation.

Blood sample was collected via abdominal aorta puncture, serum was then prepared by centrifugation of the collected blood (2000 rpm for 20 min). Serum samples were then stored at −80 °C for biochemical determinations. Femurs were dissected and filled in physiological saline and stored at −20 °C for measurement.

2.5. Assay for BMD, BMC and serum chemistry

Bone hydroxyproline was analyzed as described previously (Delport, Maas, van der Merwe, & Laurens, 2004).

The level of IL-6 in serum was measured with the commercially available enzyme-linked immunosorbent assay kit (Catalog #KAC1261; Biosource International, Inc, Camarillo, CA) according to the manufacturer's instructions.

The level of BGP in serum was measured with a competitive enzyme immune assay method, according to the manufacturer's instructions (NovoCalcin; Metra Biosystems).

The BMD and BMC of the femurs were measured using DXA equipped with appropriate software for bone density assessment in small laboratory animals as reported elsewhere (Beaupied et al., 2009).

Serum alkaline phosphatase (ALP), bone calcium (Ca), and phosphorus (P) concentrations were measured by standard colorimetric methods using commercial kits (ZhongSheng BeiKong Bio-technology and Science, PRC) and analyzed by a Cobas Integra 400 Plus automatic biochemical analyzer (Roche Diagnostics, Switzerland).

Table 3
Effect estimate.

Effect	Estimate	Std error	*t* ratio	*P*-value
X_1	0.11749	0.016829	6.9815	<0.0001
X_2	0.09053	0.016829	5.3795	0.0003
X_3	0.034282	0.016829	2.0371	0.0690
$X_1 \times X_1$	−0.029587	0.016382	−1.806	0.1011
$X_1 \times X_2$	−0.0375	0.021988	−1.7055	0.1189
$X_1 \times X_3$	−0.0125	0.021988	−0.5685	0.5822
$X_2 \times X_2$	−0.047264	0.016382	−2.8851	0.0162
$X_2 \times X_3$	−0.0375	0.021988	−1.7055	0.1189
$X_3 \times X_3$	−0.064942	0.016382	−3.9642	0.0027

2.6. Statistical analysis

Data were presented as mean ± S.D. and analyzed by one-way analysis of variance followed by Student's *t*-test for comparison of 2 groups using SPSS software Version 12.0. *P*-values of less than 0.05 were considered statistically significant.

3. Result and discussion

3.1. Fitting the models

The experimental conditions and the corresponding response values from the experimental design are given in Table 2. As observed in Table 2, treatment runs 6, 7, 8, 10, 12, 15, 16, 17, 18, 19 and 20 showed high levels of the dioscoreae polysaccharides at 1.4%, 1.5%, 1.4%, 1.5%, 1.4%, 1.4%, 1.4%, 1.4%, 1.4%, 1.4%, and 1.4%, respectively. The maximum extraction efficiency was observed in run number 7 and 10, while the minimum extraction efficiency was achieved in run number 1. The values of the coefficients in the second-order polynomial equation are presented in Table 3.

The regression equation for the extraction efficiency of dioscoreae polysaccharides (Y) was as follows:

$$\begin{aligned}Y_1 = {} & 1.401822 + 0.11749 \times X_1 + 0.09053 \times X_2 + 0.034282 \times X_3 \\ & -0.029587 \times X_1 \times X_1 - 0.0375 \times X_1 \times X_2 \\ & -0.0125 \times X_1 \times X_3 - 0.047264 \times X_2 \times X_2 \\ & -0.0375 \times X_2 \times X_3 - 0.064942 \times X_3 \times X_3 \end{aligned} \quad (2)$$

The significance of each coefficient was determined by *F*-value and *P*-value. Corresponding *P*-values suggest that, among the test variables used in this study, X_1 (extraction time), X_2 (extraction temperature), $(X_2)^2$ (extraction temperature × extraction temperature), and $(X_3)^2$ (ratio of liquid to solid × ratio of liquid to solid) are significant model terms with *P*-values of less than 0.05. Other terms are insignificant. Here, a lower value of CV (4.76) indicated a better precision and reliability of the experiments was carried out (Chen et al., 2010; Gan, Abdul Manaf, & Latiff, 2010; Kuo & Lai, 2009; Sabzali, Gholami, & Sadati, 2009). For the model fitted, the coefficient of determination (R^2), which can check the goodness of a model, was 0.92. This implied that the sample variation of 92% for the extraction efficiency of dioscoreae polysaccharides was attributed to the independent variables, and only 8% of the total variation cannot be explained by the model. From the above, it can be concluded that the developed model could adequately represent the real relationship among the parameters chosen (Table 4).

Table 4
Fit statistics for Y_1.

	Master model	Predictive model
Mean	1.305	1.305
R^2	91.76%	91.76%
Adj. R^2	84.35%	84.35%
RMSE	0.062191	0.062191
CV	4.765572	4.765572

Figs. 1A and 2A show the effects of the extraction time and temperature on the extraction yield of dioscoreae polysaccharides when the ratio of liquid to solid was fixed at 3. At this fixed ratio of liquid to solid, the maximal polysaccharides yield (1.53%) was obtained when a time of 140 min and a temperature of 100 °C were used. In Figs. 1A and 2A, increasing the extraction temperature or time within the ranges tested in this study led to an increase in the yield of polysaccharides. Therefore, the higher temperature and longer extraction time were advantageous to the extraction of dioscoreae polysaccharides. The higher mass transfer rate and solubility and the increased extraction time could be the primary factors responsible for these changes.

In Figs. 1B and 2B, when the extraction temperature was fixed at 100 °C, it was revealed that the maximum yield (1.51%) of polysaccharides was achieved when the extraction time 140 min and ratio of liquid to solid 3 were used. At all extraction times, the maximal yield of polysaccharides was attained when a extraction temperature of 100 °C was used, while ratio of liquid to solid greater than 3 caused a decrease in the extraction yield. These results imply that the quantity of the water needs to be optimized to ensure effective extraction of polysaccharides.

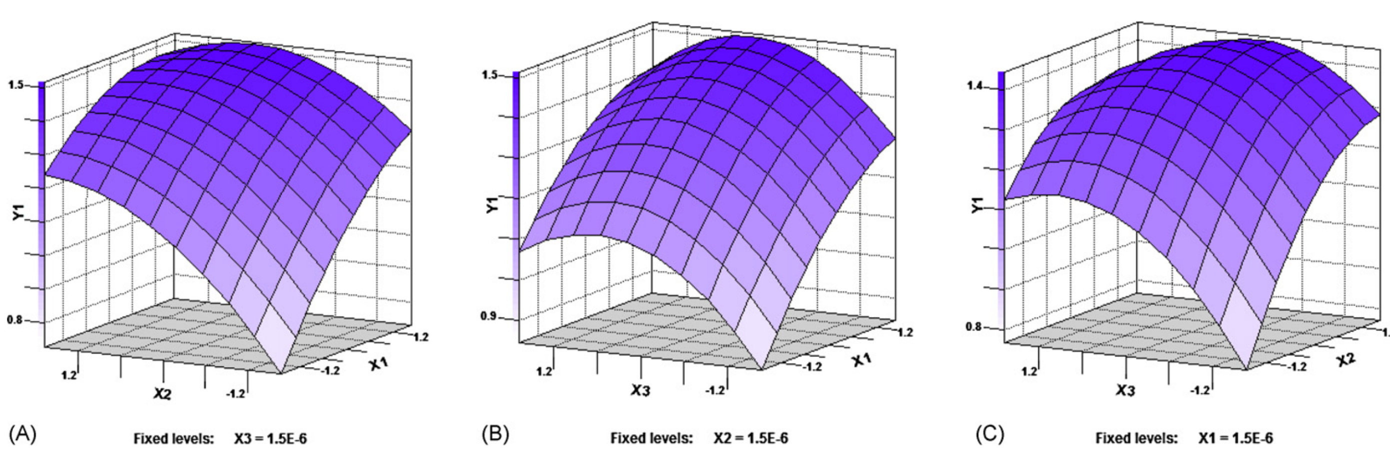

Fig. 1. (A) Three-dimensional response surface plot for the effects of extraction time (X_1) and extraction temperature (X_2) on the yield of dioscoreae polysaccharides at central level of ratio of liquid to solid (X_3); (B) three-dimensional response surface plot for the effects of extraction time (X_1) and ratio of liquid to solid (X_3) on the yield of dioscoreae polysaccharides at central level of extraction temperature (X_2); (C) three-dimensional response surface plot for the effects of extraction temperature (X_2) and ratio of liquid to solid (X_3) on the yield of dioscoreae polysaccharides at central level of extraction time (X_1).

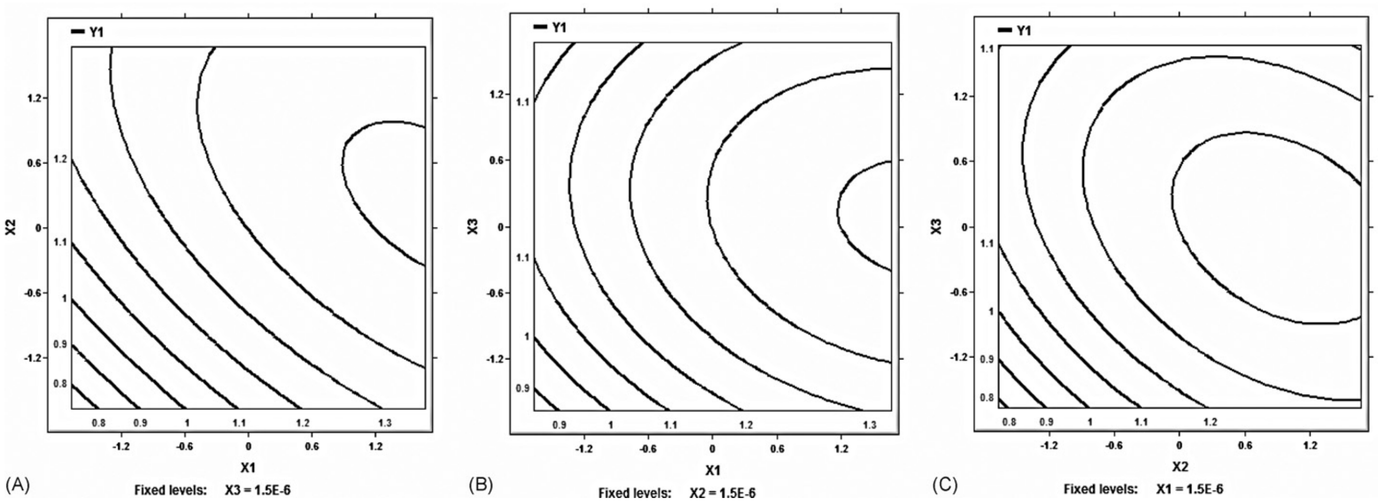

Fig. 2. (A) The contour plots of the effects of extraction time (X_1) and extraction temperature (X_2) on the yield of dioscoreae polysaccharides at central level of ratio of liquid to solid (X_3); (B) the contour plots of the effects of extraction time (X_1) and ratio of liquid to solid (X_3) on the yield of dioscoreae polysaccharides at central level of extraction temperature (X_2); (C) the contour plots of the effects of extraction temperature (X_2) and ratio of liquid to solid (X_3) on the yield of dioscoreae polysaccharides at central level of extraction time (X_1).

As shown in Figs. 1C and 2C, when a fixed extraction time of 180 min was used, the extraction yield of polysaccharides increased as the extraction temperature increased, regardless of the ratio of liquid to solid. Although the maximal extraction yield of polysaccharides for each extraction time was obtained when the ratio of liquid to solid was 3, at the concentration of the ratio greater than 3, the extraction yield of polysaccharides decreased. These findings confirmed that there is an optimal quantity of water for the effective extraction of polysaccharides. As shown in Figs. 1C and 2C, the maximum extraction of polysaccharides (1.58%) was obtained when the ratio of liquid to solid was 3 and the temperature was 100 °C.

3.2. FT-IR spectroscopy

The main absorbance bands were a broad band around 3400–3300 cm^{-1} (H bonds, OH groups), a distinct peaks at

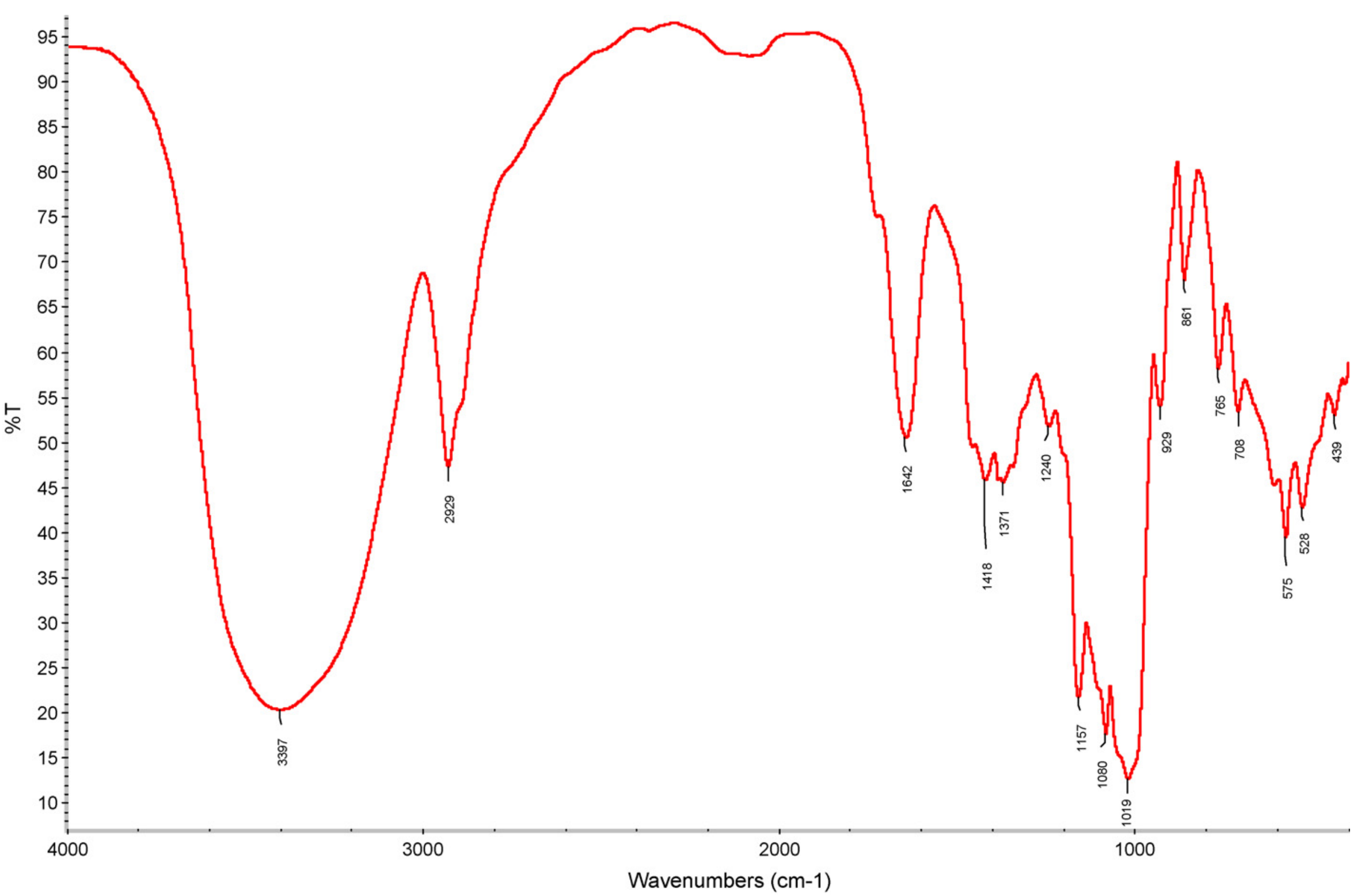

Fig. 3. Fourier transform infrared spectra of dioscoreae polysaccharides.

Table 5
Effect of dioscoreae polysaccharides on serum ALP, IL-6 and BGP levels.

Group	ALP (U/L)	IL-6 (pg/ml)	BGP (ng/ml)
1	105.91 ± 2.29	2216.25 ± 12.51	12.12 ± 0.13
2	137.12 ± 4.38**	2526.31 ± 9.44**	16.71 ± 0.19**
3	121.63 ± 2.54##	2478.92 ± 11.67##	14.25 ± 0.17##
4	114.12 ± 3.67##	2362.13 ± 14.05##	12.03 ± 0.24##

** $P<0.01$, group 1 vs group 2.
$P<0.01$, groups 3, 4 vs group 2.

Table 6
Effect of dioscoreae polysaccharides on BMD and BMC.

Group	BMD	BMC
1	0.2497 ± 0.0198	0.2407 ± 0.0211
2	0.2108 ± 0.0089**	0.2011 ± 0.0108**
3	0.2313 ± 0.0076##	0.2414 ± 0.0104##
4	0.2472 ± 0.0081##	0.2573 ± 0.0214##

** $P<0.01$, group 1 vs group 2.
$P<0.01$, groups 3, 4 vs group 2.

Table 7
Effect of dioscoreae polysaccharides on bone Hyp, Ca^{2+} and P^{3+} levels.

Group	Hyp (mg/g)	Ca^{2+} (mg/cm^3)	P^{3+} (mg/cm^3)
1	22.4 ± 1.6	353.3 ± 5.1	112.7 ± 6.9
2	17.3 ± 1.1**	305.1 ± 5.3**	93.6 ± 4.2**
3	21.8 ± 1.3##	341.7 ± 6.2##	109.6 ± 5.1##
4	23.4 ± 1.1##	361.3 ± 4.8##	122.5 ± 4.9##

** $P<0.01$, group 1 vs group 2.
$P<0.01$, groups 3, 4 vs group 2.

2929 cm^{-1} (C–H asymmetric, C–H stretch of –CH), a rising narrow sharp peak at 1642 cm^{-1} (COO–, CH_3), and a broad peak at 1371–1418 cm^{-1} (C–O stretch of polysaccharide) (Fig. 3).

FT-IR spectra in the 1700–600 cm^{-1} region give information about the main polysaccharides present in the complicated systems of polysaccharide mixture. The region at 1700–600 cm^{-1}, which is dominated by stretching vibrations of C–O, C–C, ring structures and deformation vibrations of CH_2 groups, was found to be useful for the identification of polysaccharides (Fig. 3). From Fig. 3, one can note that the polysaccharide extracts are characterized mainly by the absorbances at 1642, 1418, 1371 and 708 cm^{-1}.

3.3. Effect of dioscoreae polysaccharides on serum ALP, IL-6 and BGP levels in control and experimental animals

Table 5 depicts the effect of dioscoreae polysaccharides serum ALP, IL-6 and BGP levels in control and experimental animals. The results of the study showed a marked increase ($P<0.01$) in serum ALP, IL-6 and BGP levels in model control rats (group 2) when compared to normal control group. A marked protection against ovariectomized damage was observed by significant decrease ($P<0.05$; $P<0.01$) in serum ALP, IL-6 and BGP levels in polysaccharides-treated rats (groups 3 and 4) as compared to model rats (group 2).

3.4. Effect of dioscoreae polysaccharides on BMD and BMC

The bone indexes such as BMD and BMC were found to be decreased ($P<0.01$) significantly in model control rats (group 2) when compared to normal control animals (Table 6). A significant increase ($P<0.01$) in the BMD and BMC levels was observed in polysaccharides-treated rats after the administration of dioscoreae polysaccharides in groups 3 and 4 rats as compared with model control group.

3.5. Effect of dioscoreae polysaccharides on bone Hyp, Ca^{2+} and P^{3+} levels

Table 7 shows the effect of dioscoreae polysaccharides on bone Hyp, Ca^{2+} and P^{3+} levels in the normal control and experimental animals. In model control (group 2) animals, the levels of bone Hyp, Ca^{2+} and P^{3+} were significantly ($P<0.01$) decreased compared to normal control (group 1) animals at the end of 7 weeks. The oral supplementation of dioscoreae polysaccharides (as a 100 mg and 200 mg kg^{-1} day^{-1} dose orally for 7 weeks) to experimental rats (groups 3 and 4) resulted in significant increases in bone Hyp, Ca^{2+} and P^{3+} levels ($P<0.05$, $P<0.01$) as compared to model control (Table 7).

4. Conclusion

In this study, RSM was employed to investigate the main and the interaction effects of the variables that are important in the extraction of dioscoreae polysaccharides. The extraction time and temperature were the most important factors affecting the performance of the process. The optimum conditions were found to be extraction time of 140 min, temperature of 100 °C and the ratio of liquid to solid of 3 with the maximum yield of 1.56%. FT-IR spectra in the 1900–600 cm^{-1} region give information about the main polysaccharides present in the complicated systems of polysaccharide mixture. The pharmacological assay indicated that the extracts could still inhibit bone degeneration.

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