Carbohydrate Polymers 82 (2010) 854–860

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Optimization of extraction technology of Se-enriched *Hericium erinaceum* polysaccharides by Box–Behnken statistical design and its inhibition against metal elements loss in skull

Cheng Luo [a,*], Yin-Sheng Chen [b]

[a] *Department of Neurosurgery, The First Affiliated Hospital of Shantou University Medical College, Shantou, Guangdong Province 515041, PR China*
[b] *Department of Neurosurgery, The First Affiliated Hospital, China Medical University, Shenyang, Liaoning Province, PR China*

ARTICLE INFO

Article history:
Received 23 April 2010
Received in revised form 27 May 2010
Accepted 3 June 2010
Available online 11 June 2010

Keywords:
Box–Behnken design
Extraction
Se-enriched *Hericium erinaceum* polysaccharides
Bone loss
Skull

ABSTRACT

This article described the application of response surface methodology (RSM) to the development of a procedure for extraction of Se-enriched *Hericium erinaceum* polysaccharides. A Box–Behnken matrix was used to find optimal conditions for the procedure through a response surface study. Four variables "extraction time; extraction temperature; ratio of liquid to solid and extraction number" were regarded as factors in the optimization study. The optimal extraction conditions for the Se-enriched *H. erinaceum* polysaccharides were: extracting time 115 min, extracting temperature 98 °C, ratio of liquid to solid 4, and extracting number 4. Compared with untreated cells, administration of Se-enriched *H. erinaceum* polysaccharides improved BMC and BMD in experimental rats. These results showed that Se-enriched *H. erinaceum* polysaccharides could inhibit metal elements loss in rats' skull.

1. Introduction

Bone loss is a common and severe condition in elderly people. The pathogenesis of bone loss is unknown; however, it involves an imbalance of cartilage and bone degradation, mediated by osteoclasts, and repair, mediated by osteoblasts (OB). In osteoporosis, the decline in bone mass is favored over bone formation in the functional bone remodelling units (Ghosh & Smith, 2002; Ingram, Clarke, Fisher, & Fitzpatrick, 1993; Kouri et al., 2000). D-Galactose (D-gal) is a normal reducing sugar in the body. At the normal level, it is usually converted into glucose by galactose-1-phosphate uridyltransferase and galactokinase. However, at high levels, it can be oxidized into aldehydes and H_2O_2 in the presence of galactose oxidase (Ho, Liu, & Wu, 2003; Filioussis, Petridou, Johansson, Christodoulopoulos & Kritas, 2008; Suprabha Nair, Sindhu & Shashidhar, 2008; Zhu, Wang, Zhang, Pei, & Fen, 2008). Rodents injected with D-gal for 6–10 weeks show progressive deterioration of bone metabolism (Yokose et al., 1998), Chronic systemic D-galactose exposure induces bone loss, and brittleness of bone in mice. Moreover, D-galactose injection can also cause bone mineral homeostasis impairment (Shingu, Nagai, Isayama, Naono, & Nobunaga, 1993). The above evidence suggests that aging process induced by D-gal may contribute to some bone degeneration in animals.

Selenium is an essential trace element, which is important to develop and maintain a healthy body (Miyazaki, Koyama, Nojiri, & Suzuki, 2002). In the histological and biochemical studies of bone and articular cartilage specimens obtained from rats fed a low-selenium diet, it was shown that there were some noticeable changes in bone mineral density and in some biochemical parameters of the serum and the changes were related with the dietary selenium and the feeding duration (Pennington & Young, 1990). Snook et al. (1987) carried out histologic staining to detect mineralized and unmineralized bone and cartilage in the mice fed a selenium deficient diet and fulvic acid supplemented drinking water. Several nontoxic mushrooms contain biologically active substances. For example, polysaccharides from mushrooms have several biological actions, including anticancer (Lee, Jung, Shim, Choi, & Kim, 1981), and antigenotoxic activity (Ham, Kim, Choi, & Lee, 1997). Recent studies showed that polysaccharide isolated from the mycelium of the edible and medicinal mushroom *H. erinaceum* (Bankeraceae), also known as *H. erinaceus*, like polysaccharides from other edible mushrooms (e.g. lentinan from *Lentinus edodes* or grifolan from *Grifola frondosa*) exhibit immunostimulating and anticancer activity (Dong, Jia, & Fang, 2006; Jia, Liu, Dong, & Fang, 2006; Wang, Luo, & Liang, 2004; Zhang et al., 2006; Zhang, Cui, Cheung, & Wang, 2007). Another mode of action of *H. erinaceum* polysaccharides is cholesterol-lowering and neurite out-

* Corresponding author. Tel.: +86 574 88258290; fax: +86 574 88258290.
E-mail address: luochdc@yahoo.com.cn (C. Luo).

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doi:10.1016/j.carbpol.2010.06.005

Table 1
Experimental data and the observed responses value with different combinations of extraction time (X_1), temperature (X_2), ratio of liquid to solid (X_3), and extraction number (X_4).

Run	X_1	X_2	X_3	X_4	Y_1
1	−1	−1	0	0	12.7
2	−1	1	0	0	13.1
3	1	−1	0	0	13.2
4	1	1	0	0	13.6
5	0	0	−1	−1	13.0
6	0	0	−1	1	13.3
7	0	0	1	−1	13.2
8	0	0	1	1	13.4
9	−1	0	0	−1	12.9
10	−1	0	0	1	13.0
11	1	0	0	−1	13.2
12	1	0	0	1	13.4
13	0	−1	−1	0	13.1
14	0	−1	1	0	13.3
15	0	1	−1	0	13.3
16	0	1	1	0	13.4
17	−1	0	−1	0	13.0
18	−1	0	1	0	13.1
19	1	0	−1	0	13.3
20	1	0	1	0	13.4
21	0	−1	0	−1	13.0
22	0	−1	0	1	13.3
23	0	1	0	−1	13.2
24	0	1	0	1	13.5
25	0	0	0	0	13.2
26	0	0	0	0	13.3
27	0	0	0	0	13.2

Table 2
Fit statistics for Y_1.

	Master model	Predictive model
Mean	13.20741	13.20741
R-square	91.74%	91.74%
Adj. R-square	82.10%	82.10%
RMSE	0.082916	0.082916
CV	0.627796	0.627796

growth stimulating activity (Park et al., 2002; Yang, Park, & Song, 2003; Assefa, Beyene, & Santhanam, 2008).

In this study, we studied several aspects of bone structure and metabolism in (D-gal-induced) aged male Wistar rats fed Se-enriched *H. erinaceum* polysaccharides and compared them to control animals.

2. Materials and methods

2.1. Materials

Se-enriched *H.erinaceum* polysaccharides were kindly provided from a biotechnology company, Shanghai, China.

2.2. Experimental design

The optimization of analytical methods can be performed by two different ways: conventional univariate methodology typically represented by a process where "one variable is studied at each time", which has an easier interpretation, but requires greater amounts of

Table 3
Effects of Se-enriched *H. erinaceum* polysaccharides on BMC in rats.

Group	Total BMC (mg/mm)	Trabecular BMC (mg/mm)	Cortical BMC (mg/mm)
Control	18.11 ± 1.27	4.22 ± 0.28	9.52 ± 0.88
Model	12.13 ± 0.83[b]	2.02 ± 0.25[b]	8.01 ± 0.73[b]
Se (I)	14.85 ± 0.92[d]	2.83 ± 0.22[d]	8.48 ± 0.58[c]
Se (II)	16.31 ± 1.32[d]	3.35 ± 0.31[d]	8.91 ± 0.71[d]
Se (III)	17.25 ± 1.22[d]	3.96 ± 0.29[d]	9.35 ± 0.81[d]

[b] $P<0.05$, compared to control group.
[c] $P<0.05$, compared to model group.
[d] $P<0.01$, compared to model group.

Table 4
Effect of Se-enriched *H. erinaceum* polysaccharides on BMD in skull of rats.

Group	Total BMD (mg/cm^3)	Trabecular BMD (mg/cm^3)	Cortical BMD (mg/cm^3)
Control	831 ± 63	421 ± 32	1305 ± 61
Model	572 ± 44[b]	182 ± 19[b]	969 ± 71[b]
Se (I)	651 ± 61[d]	224 ± 20[d]	1097 ± 49[c]
Se (II)	721 ± 49[d]	293 ± 21[d]	1131 ± 62[d]
Se (III)	811 ± 73[d]	371 ± 22[d]	1271 ± 39[d]

[b] $P<0.05$, compared to control group.
[c] $P<0.05$, compared to model group.
[d] $P<0.01$, compared to model group.

Table 5
All structural mechanics index of femurs.

Group	Bone length (cm)	Bone weight (g)	Elastic deformation (mm)	Elastic load (g)	Maximum deformation (mm)	Maximum load (g)
Control	4.23 ± 0.18	0.49 ± 0.09	0.52 ± 0.06	12814 ± 1207	0.72 ± 0.10	13728 ± 917
Model	4.45 ± 0.21[b]	0.56 ± 0.08[b]	0.43 ± 0.05[b]	8301 ± 503[b]	0.61 ± 0.07[b]	8792 ± 901[b]
Se (I)	4.42 ± 0.25	0.55 ± 0.07	0.46 ± 0.04[c]	9803 ± 614[c]	0.66 ± 0.05[d]	10306 ± 793[d]
Se (II)	4.38 ± 0.31	0.51 ± 0.04[c]	0.49 ± 0.07[d]	10859 ± 405[d]	0.69 ± 0.04[d]	11205 ± 832[d]
Se (III)	4.39 ± 0.42	0.50 ± 0.09[c]	0.51 ± 0.09[d]	11842 ± 71[d]	0.73 ± 0.09[d]	12717 ± 1035[d]

[b] $P<0.05$, compared to control group.
[c] $P<0.05$, compared to model group.
[d] $P<0.01$, compared to model group.

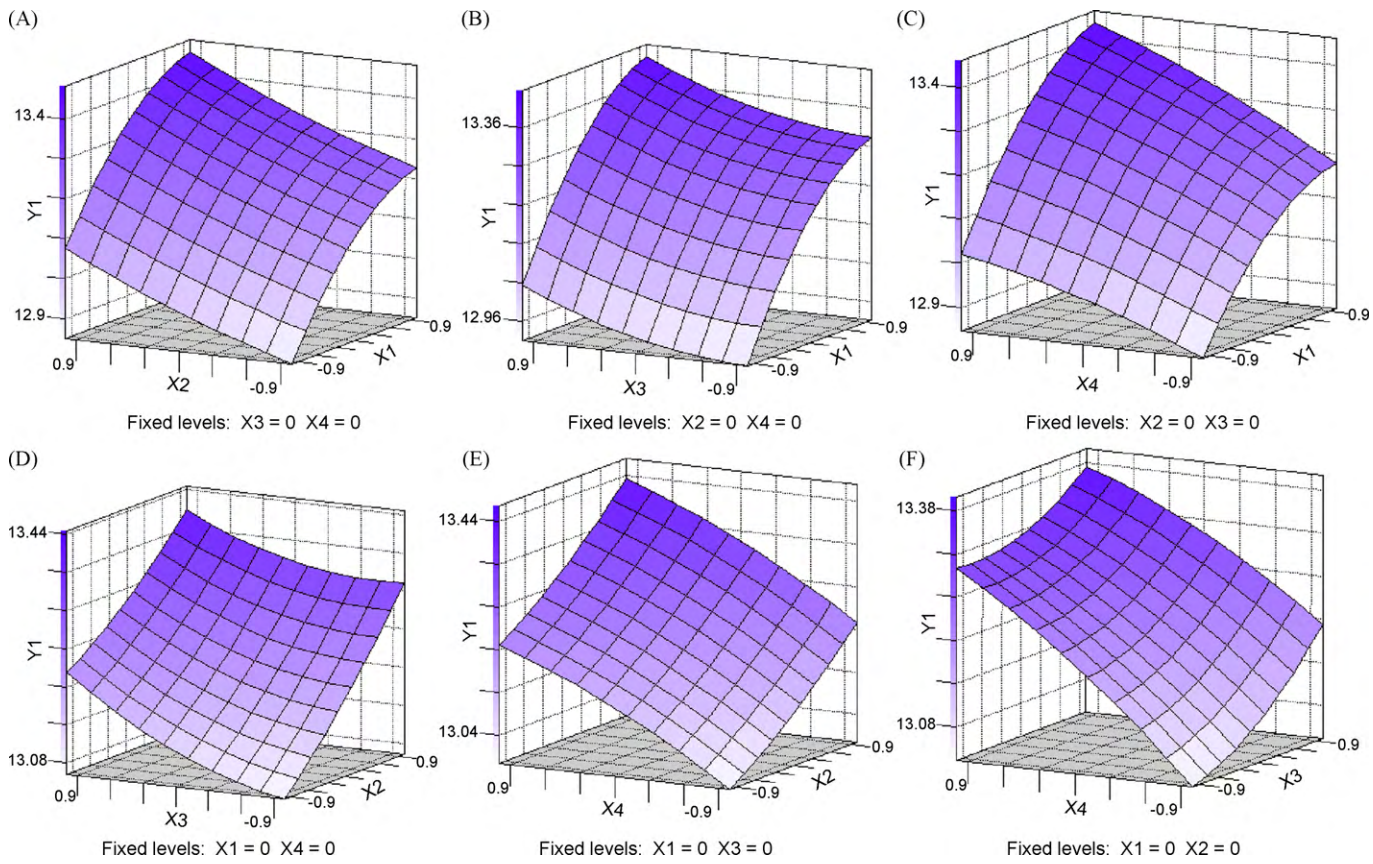

Fig. 1. (A) Response surface of effect of extraction time and temperature on extraction yield; (B) Response surface of effect of extraction time and ratio of liquid to solid on extraction yield; (C) Response surface of effect of extraction time and number on extraction yield; (D) Response surface of effect of extraction temperature and ratio of liquid to solid on extraction yield; (E) Response surface of effect of extraction temperature and number on extraction yield; (F) Response surface of effect of ratio of liquid to solid and number on extraction yield.

reagent and time to be accomplished. Besides, interactions among variables are not considered. The other way entails multivariate techniques, which are faster, more economical and effective, and allow more than one variable to be optimized simultaneously. RSM is a multivariate technique, which fit mathematically the experimental domain studied in the theoretical design through a response function (Santelli, Bezerra, SantAna, Cassella, & Ferreira, 2006). RSM is an empirical model technique devoted to the evaluation of the relationship of a set of controlled experimental factors and observed results. It requires a prior knowledge of the process to achieve statistical model. Basically, this optimization process involves three major steps, performing the statistically designed experiments, estimating the coefficients in a mathematical model, and predicting the response and checking the adequacy of the model. In this study, RSM was applied to determine the extraction conditions of Se-enriched polysaccharides from *H. erinaceus*. The effect of independent variables X_1 (extraction time, min), X_2 (extraction temperature), X_3 (solid:liquid ratio) and X_4 (extraction number) at three variation levels in the extraction process was evaluated.

2.3. Animals and diets

Wistar rats (male; $n = 50$; 115–132 g) from our laboratories were housed individually in polycarbonate cages in a constant-temperature (22 ± 2 °C) animal room with a 12 h light/dark cycle. The rats were fed a standard diet for an acclimation period of 7 days and were then divided into five groups ($n = 10$ per group) of equal average body weight: control group, model group, and Se-enriched *H. erinaceum* polysaccharides-treated groups (I, II, and III). The rats in both model group and Se-enriched *H. erinaceum* polysaccharides-treated groups (I, II, and III) received injection of D-galactose (60 mg/kg, dissolved in 0.9% saline solution, in a total volume of 1 ml/kg) intraperitoneally once a day for 40 days. The injection of D-galactose to induce aging was by an established method (Sun et al., 2007). Rats in the con-

Table 6
Effect of Se-enriched *H. erinaceum* polysaccharides on blood Ca^{2+}, P levels, and blood, skull ALP activities in rats.

Group	Ca^{2+} (mmol/L)	P (mmol/L)	ALP (U/L) blood	ALP (U/L) bone
Control	3.13 ± 0.15	3.21 ± 0.23	5.14 ± 0.37	192.31 ± 12.41
Model	3.42 ± 0.12[b]	2.31 ± 0.20[b]	8.11 ± 0.77[b]	223.27 ± 10.32[b]
Se (I)	3.39 ± 0.12	2.57 ± 0.13[d]	7.14 ± 0.46[c]	210.22 ± 12.19
Se (II)	3.33 ± 0.13[c]	2.79 ± 0.18[d]	6.33 ± 0.24[d]	201.02 ± 14.42[d]
Se (III)	3.27 ± 0.11[d]	3.01 ± 0.14[d]	5.81 ± 0.32[d]	191.53 ± 15.09[d]

[b] $P < 0.05$, compared to control group.
[c] $P < 0.05$, compared to model group.
[d] $P < 0.01$, compared to model group.

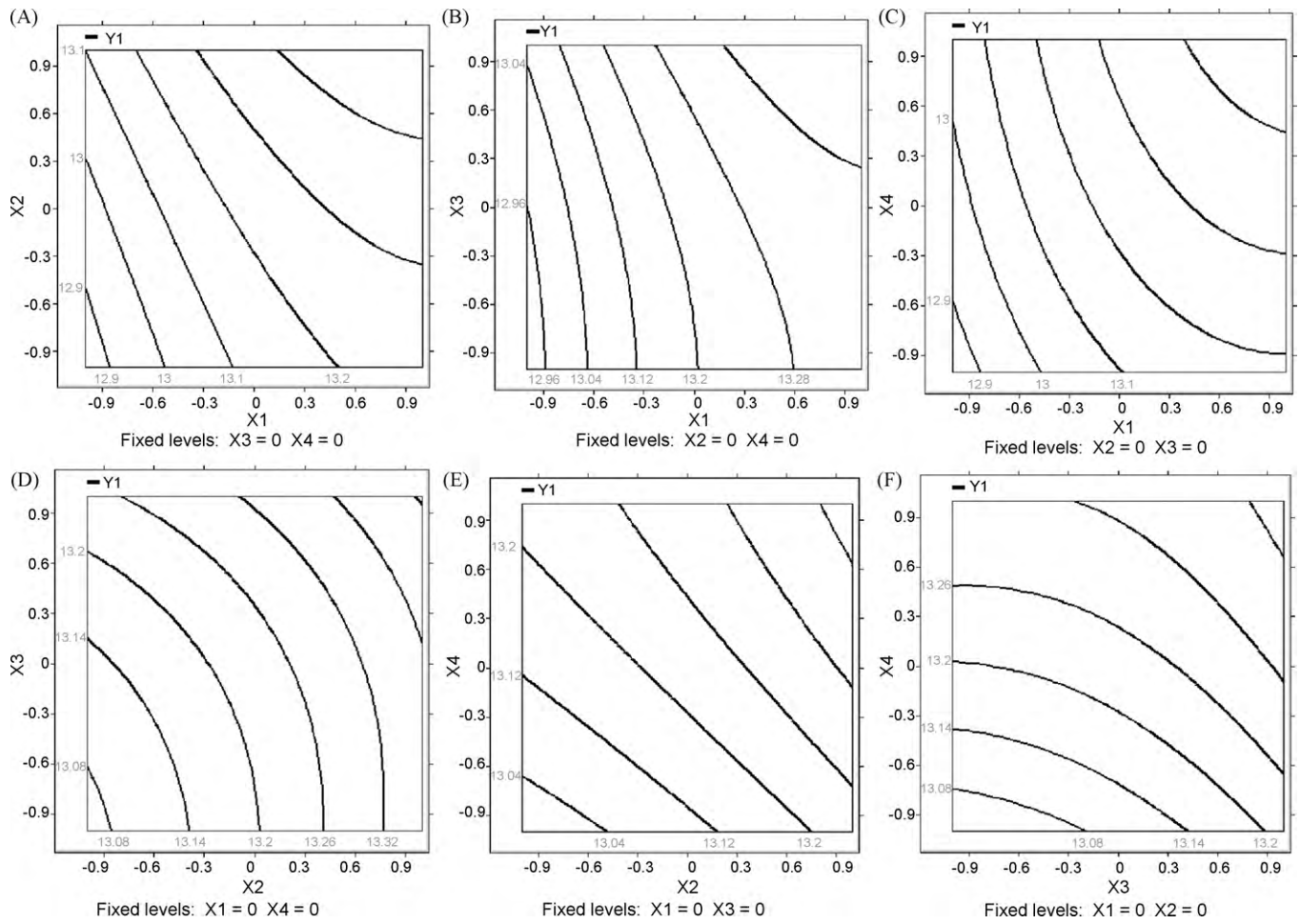

Fig. 2. (A) The contour plots of effect of extraction time and temperature on extraction yield; (B) The contour plots of effect of extraction time and ratio of liquid to solid on extraction yield; (C) The contour plots of effect of extraction time and number on extraction yield; (D) The contour plots of effect of extraction temperature and ratio of liquid to solid on extraction yield; (E) The contour plots of effect of extraction temperature and number on extraction yield; (F) The contour plots of effect of ratio of liquid to solid and number on extraction yield.

trol group received intraperitoneal injection of saline (0.9% saline, 1 ml/kg body weight) for 40 days. At the same time, rats from Se-enriched *H. erinaceum* polysaccharides-treated groups (I, II, and III) were then fed by Se-enriched *H. erinaceum* polysaccharides. Rats from control group and model group were fed a standard diet. At the end of the diet period (Day 40), blood was collected from tail tip. Rats were then sacrificed under carbon dioxide, femur and skull were dissected away from the hip joint, carefully cleaned of adherent tissue, wrapped with gauze soaked in saline and stored at 4 °C until biomechanical testing was initiated. This study was approved by the Institutional Animal Care and Use Committee of China, and animals were maintained in accordance with IACUC guidelines for the care and use of laboratory animals.

2.4. Bone mineral density (BMD) and bone mineral content (BMC) assay

The skull were cleansed off from the adhering soft tissues and stored in 75% (v/v) ethanol for a week prior to analysis. Bone mineral density (BMD) and bone mineral content (BMC) were measured at 3 mm from the skull with peripheral quantitative computed tomography (pQCT) densitometry (StratecTM XCT Research SA, Germany).

2.5. Bone biomechanical testing

Femur bones were kept at 4 °C until determination of breaking strength using a 5-kN Flexure Fixture, configured for three-point bend tests and attached to an Instron Universal Testing Machine (Shimadzu, AG-IS20KN, Japan) equipped with a 10-kN load cell (Instron, Canton, MA, USA), as previously described. The crosshead speed was 50 mm/min, and the data sampling rate was 10 samples/s. Elastic deformation, elastic load, maximum deformation and maximum load were determined in skull using Series IX, v 8.08.00 software (Instron).

2.6. Biochemical analysis

Serum Ca^{2+} and phosphor levels were measured by the EDTA titration method and malachite green colorimetric method. Serum alkaline phosphatase (ALP) activity was measured using thymol blue monophosphate method. Bone alkaline phosphatase (ALP) activity was measured using by ELISA kit (Sigma, USA).

2.7. Statistical analysis

Results were shown as means ± SEM for each group. Statistical analysis was performed using Jandel Sigma Stat (Version 2.03) statistical software. Significance of difference between two groups was evaluated using Student's *t*-test. For multiple comparisons, one-way analysis of variance (ANOVA) was used.

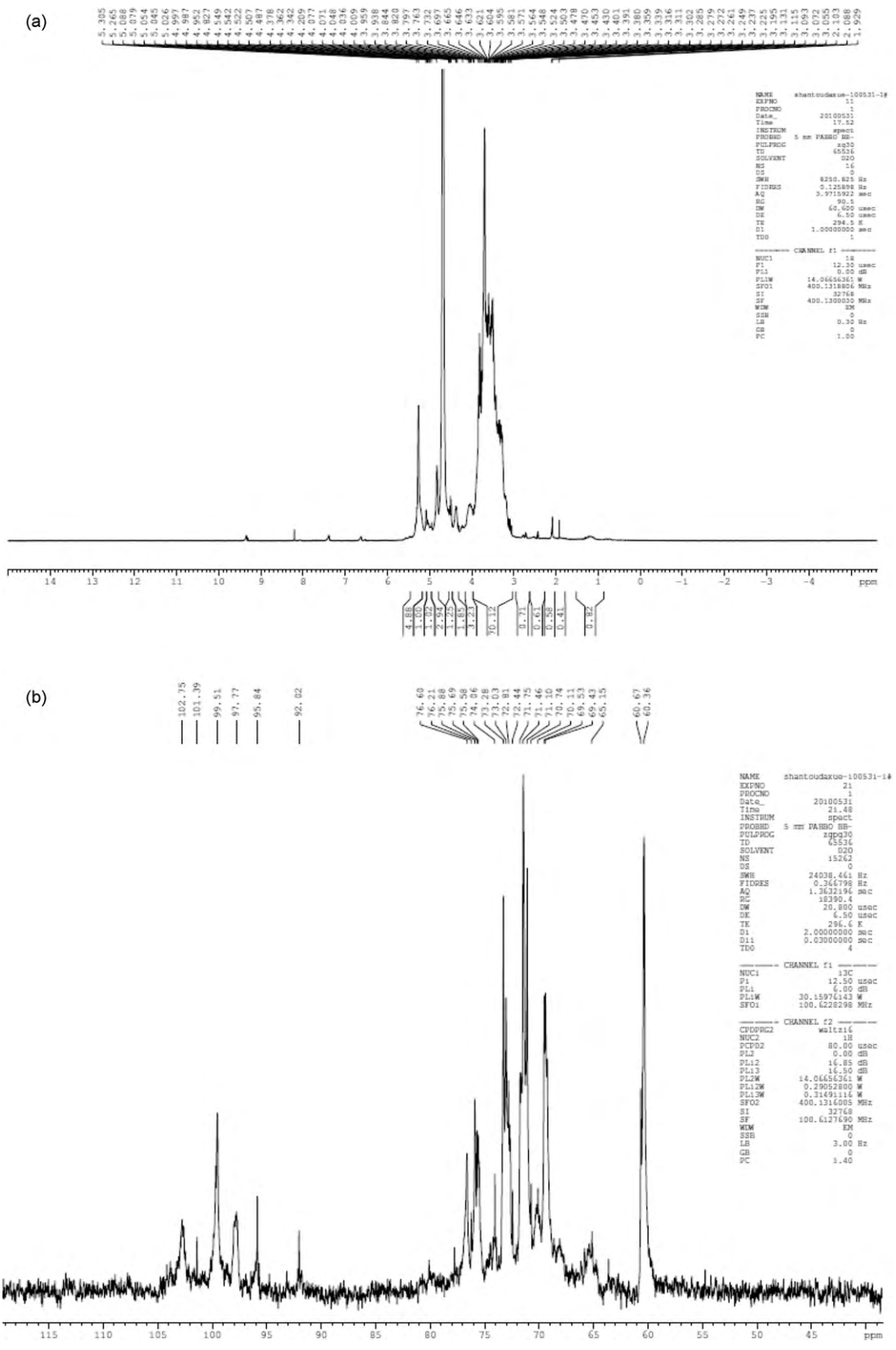

Fig. 3. (a) ^{1}H NMR analysis of Se-enriched *H. erinaceum* polysaccharides; (b) ^{1}C NMR analysis of Se-enriched *H. erinaceum* polysaccharides.

3. Results and discussion

3.1. Box–Behnken design

Table 1 shows the experimental conditions of the BBD along with the corresponding values observed for the four responses studied. Experimental data was fitted to the quadratic model by ANOVA. The ANOVA for the four responses is shown in Table 2. The analysis of variance for these models was given in Table 2. According to this model, linear terms of extraction time (X_1, $P<0.05$), extraction temperature (X_2, $P<0.01$), ratio of liquid to solid (X_3, $P<0.01$) and extraction unmber (X_4, $P<0.01$) reach significance. The result suggested that the change of extraction time, temperature, ratio of liquid to solid and extraction number had a significant effect on extract yield of polysaccharides (Figs. 1A–F and 2A–F).

The analysis showed that all of the four models were significant at 95% confidence and the lack of fit was not significant. It showed that the fitted four models were considered adequate. The model is expressed by Eq. (1).

$$Y_1 = 13.23333 + 0.191667 \times X_1 + 0.125 \times X_2 + 0.066667 \times X_3 + 0.116667 \times X_4 - 0.083333 \times X_1 \times X_1 + 0.025 \times X_1 \times X_4 + 0.016667 \times X_2 \times X_2 - 0.025 \times X_2 \times X_3 + 0.029167 \times X_3 \times X_3 - 0.025 \times X_3 \times X_4 - 0.020833 \times X_4 \times X_4 \quad (1)$$

The regressors or term incorporated in the model are those statistically tested to be significant. The coefficient of determination, R^2 for the model is 91.74%. This indicates that only 8.26% of the total variability was not explained by the regressors in the model. The high R^2 value and a small CV value indicates that the model obtained will be able to give a reasonably good estimate of response of the system in the range studied. The lack of fit test which is not significant for the model developed shows that the model adequately fits the data.

In this study, the aim of optimization was to find the conditions which give the maximum extraction yield of polysaccharides. Desirability function approach was used to achieve this goal. The software predicted the optimum extraction time, extraction temperature, ratio of liquid to solid and extraction number was 115 min, 98 °C, 4 and 4, respectively. The software predicted that the extraction yield of polysaccharides were 14.88%. In addition, NMR analysis of *H. erinaceum* polysaccharides was shown in Fig. 3a and b.

3.2. Effect of Se-enriched H. erinaceum polysaccharides on BMC and BMD in rats

pQCT was used to evaluate BMC and BMD of rats, as it can separately examine cortical and trabecular bone changes. Tables 3 and 4 show that the BMC and BMD of skull significantly decreased in total and trabecular bone, but did not change in cortical bone, compared with the control rats. Administration of Se-enriched *H. erinaceum* polysaccharides caused a significant increase in total and trabecular bone BMC and BMD in the skull, compared with model rats. These results indicated that Se-enriched *H. erinaceum* polysaccharides improved BMC and BMD of trabecular bone and decreased bone loss induced by D-galactose injection.

3.3. Effect of Se-enriched H. erinaceum polysaccharides on structural mechanics index of femurs

Rat femur bones demonstrate significant Zn-sensitive changes in bone biomechanical indices (Table 5). Bones from rats in model group exhibit significantly ($P<0.05$) larger femur length and weight, lower elastic deformation, elastic load, maximum deformation and maximum load than those from rats in the control group. Se-enriched *H. erinaceum* polysaccharides dose-dependently significantly affect these test indexs. It could be observed that lower elastic deformation, elastic load, maximum deformation and maximum load in Se-enriched *H. erinaceum* polysaccharides-treated groups were significantly enhanced, whereas femur weight was significantly reduced compared to the model group ($P<0.05$; $P<0.01$). The femur length of rats were not significantly different between Se-enriched *H.erinaceum* polysaccharides-treated groups and model group ($P>0.05$).

3.4. Effect of Se-enriched H. erinaceum polysaccharides on blood Ca^{2+}, P levels, and blood, skull ALP activities in rats

Rats received a single oral administration of Se-enriched *H. erinaceum* polysaccharides, and changes in blood Ca^{2+}, P levels, and blood, skull ALP activities were recorded (Table 6). It was noted that the significantly ($P<0.01$) decreased blood P level, and increased blood Ca^{2+} level, blood and skull ALP activities in model group could be found compared to the control group. The significantly ($P<0.05$, $P<0.01$) increased blood P level, and decreased Ca^{2+} level, blood and skull ALP activities in Se-enriched *H. erinaceum* polysaccharides-treated groups (I, II, and III) were observed in a dose-dependent pattern after administration of Se-enriched *H. erinaceum* polysaccharides.

4. Conclusion

The Box–Behnken design (BBD) was found to be a valuable tool to estimate the effect of the extracting time, extracting temperature, rate of solid to liquid and extraction number for optimising the extraction of Se-enriched *H. erinaceum* polysaccharides. Analysis of variance shows that the regression model for extraction of Se-enriched *H. erinaceum* polysaccharides were statistically good with a significance level of $P<0.0001$ and the models had no significant ($P>0.05$) lack of fit. Thus, well-fitting models for the extraction of Se-enriched *H. erinaceum* polysaccharides were successfully established. The optimal conditions for the extraction of Se-enriched *H. erinaceum* polysaccharides were: extracting time 115 min, extracting temperature 98 °C, ratio of liquid to solid 4, and extracting number 4. The experimental values were shown to be in agreement with those predicted, thus indicating adequacy of the fitted models. This study showed that administration of Se-enriched *H. erinaceum* polysaccharides improved BMC and BMD in experimental rats. This study suggests that Se-enriched *H. erinaceum* polysaccharides could inhibit metal element loss in skull.

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