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Optimization of ultrasonic-assisted extraction of polyphenol from Areca nut (*Areca catechu L.*) seeds using response surface methodology and its effects on osteogenic activity

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ABSTRACT

Areca nut (*Areca catechu L.*) seeds are rich in polyphenols, while few studies focused on it. This study was designed to obtain the maximum extraction yield of areca nut seed polyphenol (ACP). An ultrasonic-assisted extraction method optimized by response surface methodology (RSM) was established to extract ACP. Under the optimal conditions (ultrasonic power of 87 W, ethanol concentration of 65%, extraction temperature of 62℃, and extraction time of 153 min), the actual extraction yield of ACP was 139.62 mg/g. Then we investigated the effects of ACP on the proliferation, differentiation and mineralization of MC3T3-E1 pre-osteoblasts. Results suggested that ACP notably promoted the proliferation of MC3T3-E1 cells without cytotoxicity, and the contents of collagen type I (COL-I) and osteocalcin (OCN) were rising. Meanwhile, the alkaline phosphatase (ALP) activity and mineralized nodules were enhanced. These findings demonstrated that ACP could induce the proliferation, differentiation and mineralization of osteoblasts *in vitro*. This work provided a certain experimental basis for the developing and utilization of polyphenols from Areca nut seeds.

1. Introduction

Areca nut (*Areca catechu L.*) is a crucial cash crop in the tropics. In recent years, the Areca nut industry has developed rapidly and has become an important pillar of economic development in Hainan Island [1]. The prime active components in Areca nut are alkaloids and phenolic compounds. However, studies have fully proved that alkaloids are the main cause of Areca nut addiction and carcinogenesis [2]. As another functional component of Areca nut, phenolic compounds are rarely studied and reported at present. The phenolic compounds have the highest content in Areca nut seeds, accounting for about 30% [3]. They have antibacterial, antioxidant, hypoglycemic, antithrombotic and other biological activities [4]. Our previous study reported that the polyphenol extracted from Areca nut seeds could alter the gut microbiome and immune system to improve osteoporosis in osteoporosis rats induced by estrogen deficiency [5]. Lee et al. [6] found that the phenolic compounds in Areca nut can significantly inhibit skin aging and inflammation. Wetwitayaklung et al. [7] revealed that the phenolic compounds in Areca nut seeds had the strongest free radical scavenging ability, which had great development potential in the food or medical field. Hence, it is vital to establish an effective, safe, and environmentally friendly extraction technology in the development of polyphenols from Areca nut.

Ultrasound is an emerging green, safe and low-cost technology that is widely used in the food industry, such as modification, extraction, degassing, and emulsification [8,9]. Ultrasonic-assisted extraction can destroy the tissue or cells by the "cavitation effect", enhancing the mass transfer rate and facilitating the release of polyphenol [10,11]. Nowadays, the ultrasonic wave has been extensively adapted to assist the extraction of active substances from plant materials. Compared with traditional methods like Soxhlet extraction, hot reflux extraction and immersion extraction, ultrasonic-assisted extraction has attracted research interest recently because of its characteristics of higher yield, shorter processing time and less solvent [12,13]. Several physical-assisted extraction polyphenols methods, such as microwave-assisted extraction, high-pressure-assisted extraction, and supercritical carbon dioxide extraction, have the disadvantages of longer extraction time, lower extraction efficiency, higher power consumption, and more organic solvent pollution than ultrasonic-assisted extraction [14]. Thus, these methods limit the extraction of polyphenols from hard seeds. The

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benefits of ultrasonic-assisted extraction include promoting the hydration of dried seeds, destroying the cell walls to increase the mass transfer and causing no changes in the structure and function of polyphenols, which significantly improves the efficiency of extraction [15]. Belwal et al. [16] found that under the optimal ultrasonic-assisted extraction conditions, significantly higher polyphenolic contents and antioxidant activity were recorded than conventional extraction. Serna et al. [17] reported that the extraction yield was as high as 85%, at a markedly lower energy requirement using ultrasound-assisted extraction instead of conventional solid–liquid extraction. The antioxidant activity of polyphenol from citrus peels using ultrasound-assisted extraction was higher by about 28% than that when using microwave-assisted extraction [18].

Postmenopausal osteoporosis is a common chronic disease with a severe physical and financial impact on patients. At present, the clinical treatment mainly uses metabolic drugs that inhibit bone resorption and promote bone formation to treat osteoporosis. However, long-term clinical treatment has brought serious side effects, such as hypercalcemia, breast cancer, uterine bleeding and other adverse reactions [19]. Numerous reports have proved that functional components originating from organisms can be safe and reliable routes for disease treatment and prevention.

To our knowledge, ultrasonic-assisted extraction using ethanol to extraction ACP has not been reported before, and there is no report about ACP on bone metabolism *in vitro*. Herein, ultrasonic-assisted extraction of ACP was established to develop and optimize via response surface methodology, then the effects of ACP on pre-osteoblast proliferation, differentiation, and mineralization were examined using mouse pre-osteoblasts *in vitro*.

2. Materials and methods

2.1. Materials and reagents

The Areca nut seeds were acquired from Hainan Huachuang *Areca catechu* Research Institute (Hainan, China). The gallic acid standard was acquired from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). MC3T3-E1 cell line, purchased from American Type Culture Collection (Rockville, USA). Alpha-minimum essential medium (α-MEM), phosphate-buffered saline (PBS), Fetal bovine serum (FBS), trypsin and Penicillin-Streptomycin Nystatin Solution were purchased from Shanghai Lifei Biotechnology Co., Ltd. (Shanghai, China). Alizarin Red S solutions, cetylpyridinium chloride, β-sodium glycerophosphate, and L-Ascorbic acid were obtained from Sigma-Aldrich Co. (Shanghai, China). Alkaline Phosphatase Assay Kit and BCA Protein Assay Kit were obtained from Shanghai Biyuntian Biotechnology Co., Ltd. (Shanghai, China). Collagen Type I (COL-I) enzyme-linked immunosorbent assay (ELISA) Assay kit and Osteocalcin ELISA Assay Kit were obtained from Nanjing Jiancheng Biological Engineering Research Institute (Nanjing, China). Other chemicals were analytical grade.

2.2. Extraction of ACP

The Areca nut seeds were ground and sieved with a 100-mesh sieve. The powder (0.2 g) was mixed with ethanol aqueous solution (50 mL) in a 50 mL centrifuge tube. The suspension was extracted by a 40 kHz ultrasonic cleaner (XO-5200DTD, Nanjing Xianou Instruments Manufacture Co., Ltd., Jiangsu, China). Then the crude extract was centrifuged at 3500 rpm for 10 min (Hunan Xiang Yi Laboratory Instrument Development Co., Ltd., Hunan, China). After that, the supernatant was filtered through a 0.45 μm filter membrane, the filtrate was collected for further study. The extraction yield (mg/g) of ACP was figured out by equation (1):

$$Y = \frac{C \times d \times V}{m} \quad (1)$$

where, Y is the extraction yield (mg/g) of ACP; C is the concentration (mol/L) of ACP; d is the dilution factor; V is the volume (L) of the sample, m is the mass (g) of the sample.

2.3. Single-factor experiment

The single-factor extraction experiment was performed under the following conditions: the ultrasonic power of 40–180 W, ethanol concentration of 50–90%, extraction temperature of 40–80℃, and extraction time of 0.5–3 h. The experiment design is shown in Table 1.

2.4. Response surface design

The four-factor and three-level experimental designs were determined on the basis of the single-factor experimental design. Ultrasonic power (A), ethanol concentration (B), extraction temperature (C), and extraction time (D) were the independent variables, the extraction yield of ACP was the dependent variable. The experiment was optimized by Box-Bohnken (B-B) design. Table 2 exhibited the factors and coding levels.

2.5. Measurement of ACP content

The ACP content was measured by Folin-Ciocalteu (FC) reagent method selecting gallic acid as a standard, following the second method of GB/T 8313–2018 with slight modifications. Using the gallic acid standard curve [$A = 0.005C + 0.0861$, $r^2 = 0.9968$] to calculate the ACP content. The outcome was presented in mg of gallic acid equivalent per g of Areca nut seed powder.

2.6. Culture of pre-osteoblast MC3T3-E1

Removed the old medium from the culture flask, added PBS to clean cell fragments and metabolites, then removed PBS and added 2 mL trypsin containing 0.25% EDTA and digested 2 min. After removing the trypsin, 2 mL of the complete medium (α-MEM including 10% fetal bovine serum and 100 U/mL streptomycin-penicillin) was added to the culture flask to pipette the cells until they are completely suspended. Finally, 1 mL of the medium including cells was absorbed and shifted to a T25 culture flask containing 7 mL of complete medium, and cultured in a 37℃, 95% humidity, 5% carbon dioxide incubator.

2.7. Proliferation assay of pre-osteoblast MC3T3-E1

Cells at a density of 2×10^4 cells/mL were added into a 96-well plate at 100 μL per well and incubated for 1 day. Then the medium containing ACP (0, 25, 50, 100, 150 μg/mL) was added and cultured for 2 days. The proliferation of cells was measured by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) method. Added 20 μL, 5 mg/mL MTT to each well and cultured for 4 h in an incubator. After that, removed the supernatant and added 150 μL of DMSO to each well, then shaken for 10 min. The absorbance (A490 nm) was detected on a microplate reader.

Table 1
Single-factor experimental design.

Single factor	Power (W)	Concentration (%)	Temperature (℃)	Time (h)
Power	40, 80, 120, 180	120	120	120
Concentration	70	50, 60, 70, 80, 90	70	70
Temperature	70	70	40, 50, 60, 70, 80	70
Time	1	1	1	0.5, 1, 1.5, 2, 3

Table 2
Factors and their coding levels in experimental design for RSM.

Factors	Levels		
	−1	0	1
A: Ultrasonic power (W)	40	80	120
B: Ethanol concentration (%)	60	70	80
C: Extraction temperature (℃)	60	70	80
D: Extraction time (h)	1.5	2.0	3.0

2.8. Determination of alkaline phosphatase (ALP) activity

MC3T3-E1 cells were laid in 24-well plates at a density of 2×10^4 cells/mL, with 1 mL in each well. After incubated in the incubator for 1 day, the medium was substituted with α-MEM differentiation medium containing 10 mM β-sodium glycerophosphate, 50 μg/mL ascorbic acid, 10% fetal bovine serum, 100 U/mL streptomycin-penicillin, and ACP (0, 25, 50, 100, 150 μg/mL). The medium was replaced every 2 days. The cells were gathered on the 7th day, the medium was discarded, and washed twice with precooled PBS. Then added 300 μL 1% TritonX-100 lysate to each well, and the cells were lysed at 4℃ for 20 min. After that, centrifuged at 12,000 r/min 4℃ and collected the supernatant. The relative enzyme activity was detected referring to the directions of the alkaline phosphatase assay kit. The total cell protein was detected by the BCA protein assay kit.

2.9. Determination of COL-I and osteocalcin (OCN) content

The cell culture was the same as 2.8, and the supernatant was gathered on the 7th day and 14th day, respectively. The content of COL-I was measured referring to the indications of the COL-I assay kit. And the content of OCN was determined by referring to the indications of the OCN assay kit.

2.10. Determination of mineralized nodules

The medium of cell culture and differentiation medium was the same as 2.8. Changed the medium every 2 days and detected on the 17th day. Then removed the supernatant, and washed twice using PBS, then the cells were fixed with 300 μL 4% neutral formaldehyde for 15 min. After that, washed twice with precooled PBS, adding 300 μL 1% alizarin red S solution to stain for 30 min at 37℃ in the darkness. Finally, washed the cells with running water 3 times and observed.

After recording, added 300 μL of 100 mM cetylpyridinium chloride solution to each well, and reacted at 37℃ for 15 min in the darkness for semi-quantitative detection. Absorbed 100 μL of the supernatant from each well and added it to the 96-well plate. The absorbance was tested at 570 nm.

2.11. Statistical analysis

All the experiments were repeated for 3 times, the data were shown in mean ± standard deviations (SD). The data were analyzed by ANOVA followed by Duncan's post hoc testing SPSS 25.0. The significance level was designated as $p < 0.05$. The outcomes derived from the RSM design were computed through Design-Expert 13.0 software.

3. Results

3.1. Single-factor experiment

3.1.1. Effect of ultrasound power on the extraction yield of ACP

As shown in Fig. 1a, ultrasonic power played a significant role in enhancing the extraction yield of ACP. With the elevation of ultrasonic power, the yield initially increased and then decreased at higher power (120 W). The extraction yield reached the peak (122.45 ± 0.87 mg/g) at 80 W. Since the cavitation effect generates shear force, mechanically breaks the cell walls and conduces to the migration of substances, the yield was increased after ultrasonic treatment [20]. As the ultrasonic

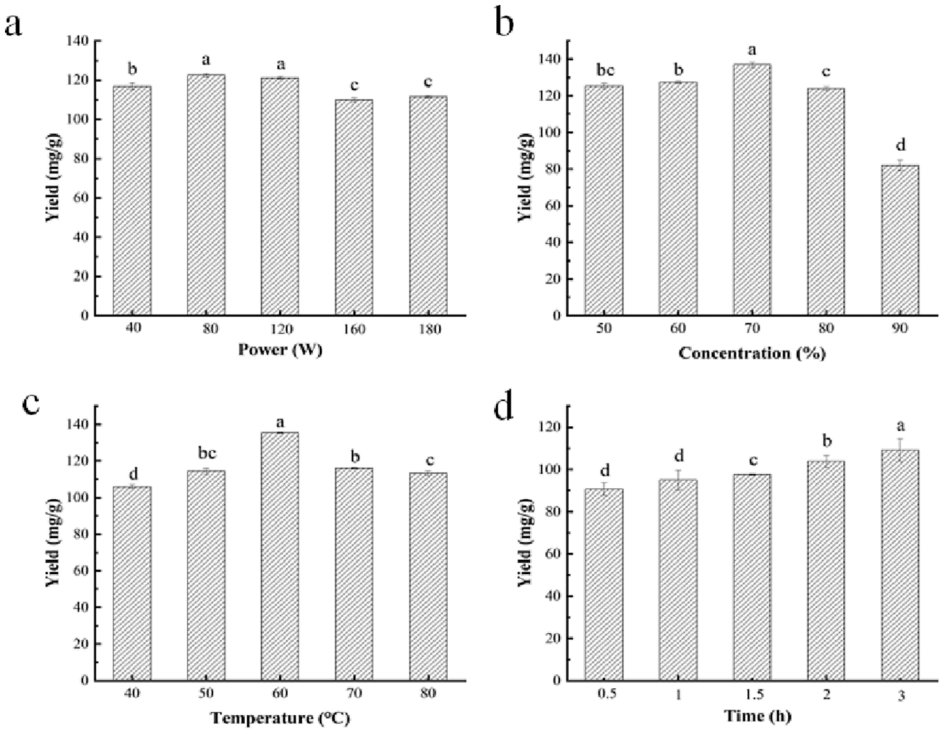

Fig. 1. Effect of ultrasonic power (a), ethanol concentration (b), temperature (c), and time (d) on the extraction yield of ACP. Values are expressed as means ± SD (n = 3). Different lowercase letters (a, b, c, and d) in the same figure indicate a statistically significant difference ($p < 0.05$).

power kept increasing, the extraction yield reduced. A high power could generate multiple bubbles, which hindered the transmission of ultrasonic waves [21]. In addition, it could also be explained by the damage of the polyphenol structure, the dissolution of impurities and the reduction of active components caused by the significant high power [22,23]. Taken together, 80 W was considered to be the optimal ultrasonic power.

3.1.2. Effect of ethanol concentration on the extraction yield of ACP

As exhibited in Fig. 1b, with the rise of ethanol concentration between 50% and 70%, the yield was rising, while the yield declined as the concentration exceeded 70%. The extraction yield reached the highest value (136.95 ± 1.32 mg/g) at 70%. The trend was corresponding to the report of Wang et al. [24]. The hydroxyl groups of phenolic compounds could interact with the hydroxyl, amidogen and carboxyl groups to form stable compounds in proteins and polysaccharides [25,26]. When the concentration of ethanol was increased, non-covalent interactions like hydrogen bonds and hydrophobic interactions were destroyed [27], contributing to the dissolution of ACP. Afterward, the concentration had an adverse influence on the yield of ACP. This result may be ascribed to the transformation of solvent polarity as the concentration was continuously increased [28]. Moreover, this behavior resulted from the competitive interactions between ethanol, water, and polyphenol molecules [29]. As a result, the optimum ethanol concentration is 70%.

3.1.3. Effect of extraction temperature on the extraction yield of ACP

Within 40–80℃, the extraction yield increased substantially and then decreased significantly when the temperature was over 60℃ (Fig. 1c). The yield reached a maximum (135.28 ± 0.29 mg/g) at 60℃. It indicated that elevated temperature exerted an adverse effect on the extraction of ACP. The hydroxyl groups of phenols are prone to oxidation and condensation reactions, which was key responsible for the extraction yield of ACP [30]. Furthermore, when the temperature exceeded the boiling point of ethanol, the evaporation of ethanol was accelerated. Consequently, 60℃ was chosen to be the best extraction temperature.

3.1.4. Effect of extraction time on the extraction yield of ACP

According to Fig. 1d, the yield showed an increasing time-dependent effect. Though the high yield of ACP was relevant to the extension of extraction time, immoderate extraction time should be averted because of the thermal degradation of ACP [31]. For instance, while the tea polyphenols were extracted with ethanol over 60 min, the total content was reduced [32]. Additionally, long-time extraction would increase the cost. Therefore, 3 h was selected to be the optimal extraction time.

3.2. Optimization of ACP extraction process

3.2.1. RSM model fitting

According to the analysis of the single-factor experiment, the four-factor and three-level test was proceeded by RSM. A total of 29 experimental combinations were presented in Table 3. The results were fitted via multiple regression using Design-Expert, and the relationship between the extraction yield of ACP and variables was expressed by the following Equation (2):

$$Y = 136.12 + 2.89A - 6.28B - 4.57C + 2.60D + 0.4150AB - 3.00AC - 0.4175CE - 0.3750BCE - 0.2925BD + 0.4200CD - 5.33A^2 - 5.92B^2 - 2.52C^2 - 6.06D^2 \quad (2)$$

where Y is the predicted value of the extraction yield of ACP; A, B, C, and D are coding values of the independent variables as described above.

3.2.2. RSM analysis of the extraction yield of ACP

The data were analyzed by ANOVA to assess the significance of these factors and their interactions in this model. In Table 4, the model was remarkably significant ($p < 0.0001$). The credible predictive value ($R^2 = 0.9412$) showed a high correlation between the predicted value and the experimental value. The adjusted coefficient of determination was $R^2_{Adj} = 0.8623$. The difference between R^2 (0.9412) and R^2_{Adj} (0.8623) was<0.2, suggesting reasonable agreement between them. The absence

Table 3
Box-Behnken design and resultant responses.

Run	A: Power (W)	B: Concentration (%)	C: Temperature (℃)	D: Time (h)	Y: Yield (mg/g)
1	−1	−1	0	0	128.95
2	1	−1	0	0	131.12
3	−1	1	0	0	114.12
4	1	1	0	0	117.95
5	0	0	−1	−1	128.62
6	0	0	1	−1	116.78
7	0	0	−1	1	133.78
8	0	0	1	1	123.62
9	−1	0	0	−1	120.12
10	1	0	0	−1	120.62
11	−1	0	0	1	125.62
12	1	0	0	1	130.45
13	0	−1	−1	0	138.12
14	0	1	−1	0	128.95
15	0	−1	1	0	129.12
16	0	1	1	0	118.45
17	−1	0	−1	0	125.12
18	1	0	−1	0	139.78
19	−1	0	1	0	124.45
20	1	0	1	0	127.12
21	0	−1	0	−1	129.12
22	0	1	0	−1	115.95
23	0	−1	0	1	134.62
24	0	1	0	1	120.28
25	0	0	0	0	135.28
26	0	0	0	0	133.95
27	0	0	0	0	135.95
28	0	0	0	0	137.45
29	0	0	0	0	137.95

Table 4
Analysis of variance of RSM of extraction yield of ACP.

Source	Sum of Squares	df	Mean Square	F Value	Prob > F
Model	1416.12	14	101.15	16.01	< 0.0001***
A-Power (W)	100.11	1	100.11	15.84	0.0014**
B-Concentration (%)	473.14	1	473.14	74.87	< 0.0001***
C-Temperature (℃)	250.53	1	250.53	39.64	< 0.0001***
D-Time (h)	80.91	1	80.91	12.80	0.0030**
AB	0.6889	1	0.6889	0.1090	0.7462
AC	35.94	1	35.94	5.69	0.0318*
AD	0.6972	1	0.6972	0.1103	0.7447
BC	0.5625	1	0.5625	0.0890	0.7698
BD	0.3422	1	0.3422	0.0542	0.8194
CD	0.7056	1	0.7056	0.1116	0.7432
A^2	184.39	1	184.39	29.18	< 0.0001***
B^2	226.98	1	226.98	35.92	< 0.0001***
C^2	41.21	1	41.21	6.52	0.0230*
D^2	238.34	1	238.34	37.71	< 0.0001***
Residual	88.48	14	6.32		
Lack of Fit	77.92	10	7.79	2.95	0.1543
Pure Error	10.56	4	2.64		
Cor Total	1504.60	28			

*, the difference is significant at 0.05 level ($p < 0.05$); * *, the difference is significant at 0.01 level ($p < 0.01$); ***, the difference is significant at 0.001 level ($p < 0.001$)

of fit term ($p = 0.1543 > 0.05$) was not significant and the coefficient of variation (CV = 1.97%) was<5%, indicating the data had fine fit and reproducibility. Moreover, A, B, C, D, AC, A^2, B^2, and D^2 had a remarkable effect ($p < 0.0001$) on the yield of ACP, showing the rationality of the single-factor experiment.

Fig. 2 depicted three-dimensional (3D) surface plots and contour plots those reflected the influence of each factor and the interaction between any two factors on the yield of ACP. In the 3D surface plot, the steeper of the surface plot, the more obvious the interaction between the variables. The contour plot is the bottom projection of the response surface and represents the interaction between two factors. If the contour plot tends to be elliptical, the interaction between the two factors is significant [33,34]. In contrast, if it tends to circles, the interaction is not significant. With the increase of four factors, the yield of ACP tended to increase, but when the four factors exceeded certain values, their influence on the yield of ACP showed a downward tendency.

Ultrasonic power and extraction temperature exhibited notable interaction ($p < 0.05$) on the yield of ACP (Fig. 2b). While the temperature was lower than 70℃, the yield was rising markedly with the rise of ultrasonic power. The powerful cavitation effect could account for this result [1]. However, the yield tended to decrease with the elevation of power as the extraction temperature was over 75℃. Excessive power led to the degradation of phenolic compounds [3], thereby reducing the yield of ACP. Except for the above two factors, the interaction between the other two factors is not remarkable ($p > 0.05$). Comparing the F-values of varied factors and the slopes of the 3D response surface plots, we inferred the most important factor affecting the yield was ethanol concentration, followed by extraction temperature, ultrasonic power, and extraction time.

3.2.3. Verification of optimally predicted extraction yield of ACP

Based on the statistical analysis with Design-Expert 13.0, the optimal extraction conditions of ACP were obtained: ultrasonic power 87.12 W, ethanol concentration 64.58%, extraction temperature 61.96℃, extraction time 152.82 min, and the yield of ACP 139.878 mg/g. For the feasibility of the experiment, the actual conditions were: ultrasonic - power of 87 W, ethanol concentration of 65%, extraction temperature of 62℃, and extraction time of 153 min. The verification assay was carried out based on the above conditions, representing the yield of ACP was 139.62 mg/g with a small deviation (RSD = 0.18%) compared with the model prediction. Consequently, the model had good veracity and reproducibility.

3.3. Effects of ACP on MC3T3-E1 cells proliferation

To verify the influence of ACP on pre-osteoblast proliferation, the MC3T3-E1 cells were treated with different concentrations of ACP (25–150 μg/mL). From Fig. 3a, the proliferation rate of MC3T3-E1 cells rose with the increase of the concentration of ACP (0, 25, 50, 100, 150 μg/mL), and the proliferation rate of the 100 μg/mL and 150 μg/mL groups were the highest, with no remarkable difference ($p > 0.05$). These consequences manifested ACP can significantly induce the proliferation of MC3T3-E1 pre-osteoblast cells without cytotoxicity. Similarly, Patel et al. [35] reported that the primary calvarial osteoblasts had a significant increase in proliferation after the polyphenol extracted from S.cordifolia was treated. And low concentration quercetin could significantly increase (143.14%) the number of osteoblasts compared with the untreated group [36].

3.4. Effects of ACP on ALP activity

When induced by β-glycerophosphate and L-ascorbic acid, the MC3T3-E1 cells, as the precursor of osteoblasts, can differentiate into osteoblasts [37]. To evaluate the effect of ACP on cell differentiation, we measured the

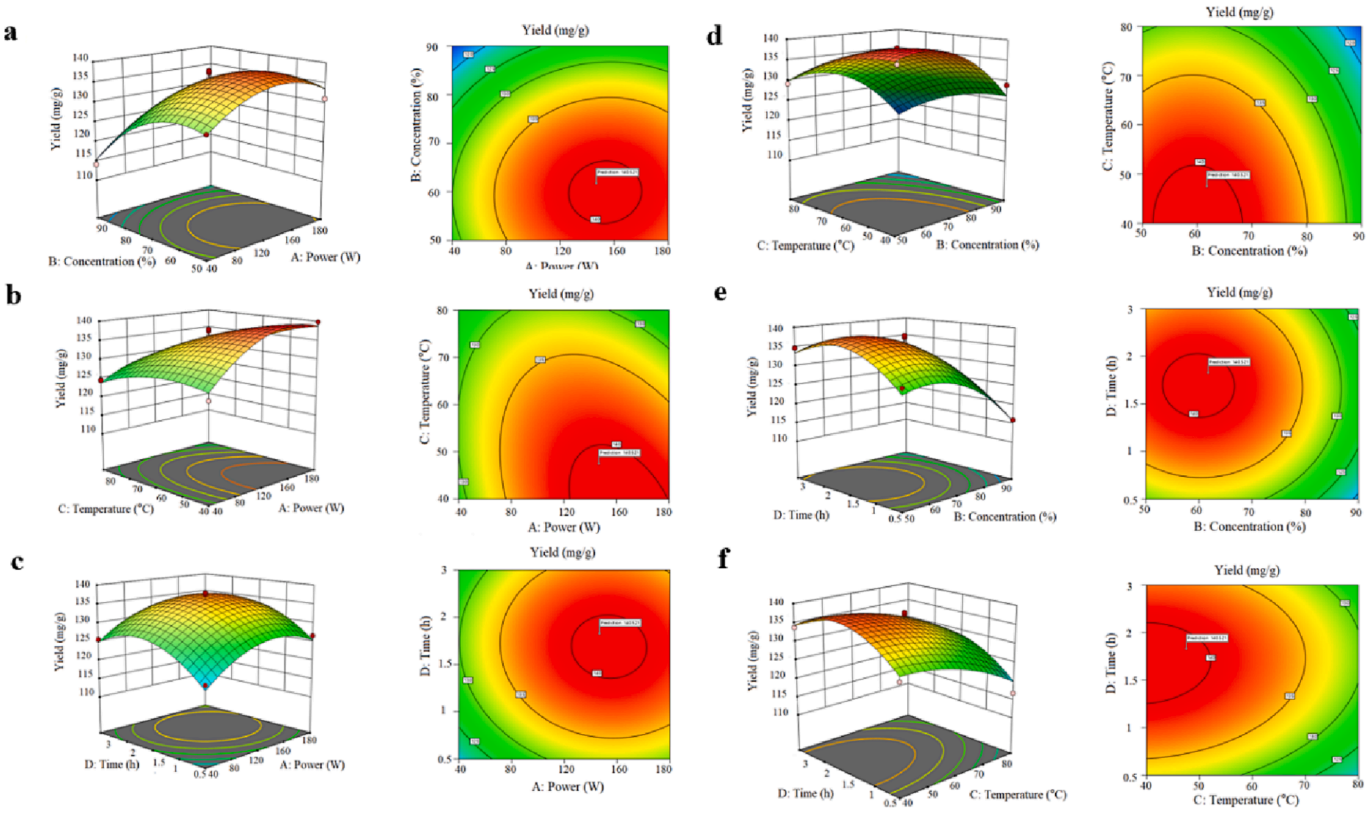

Fig. 2. Response surface plots and contour plots for interaction between various factors on the yield of ACP, ultrasonic power and ethanol concentration (a), ultrasonic power and extraction temperature (b), ultrasonic power and extraction time (c), ethanol concentration and extraction temperature (d), ethanol concentration and extraction time (e), and extraction temperature and extraction time (f).

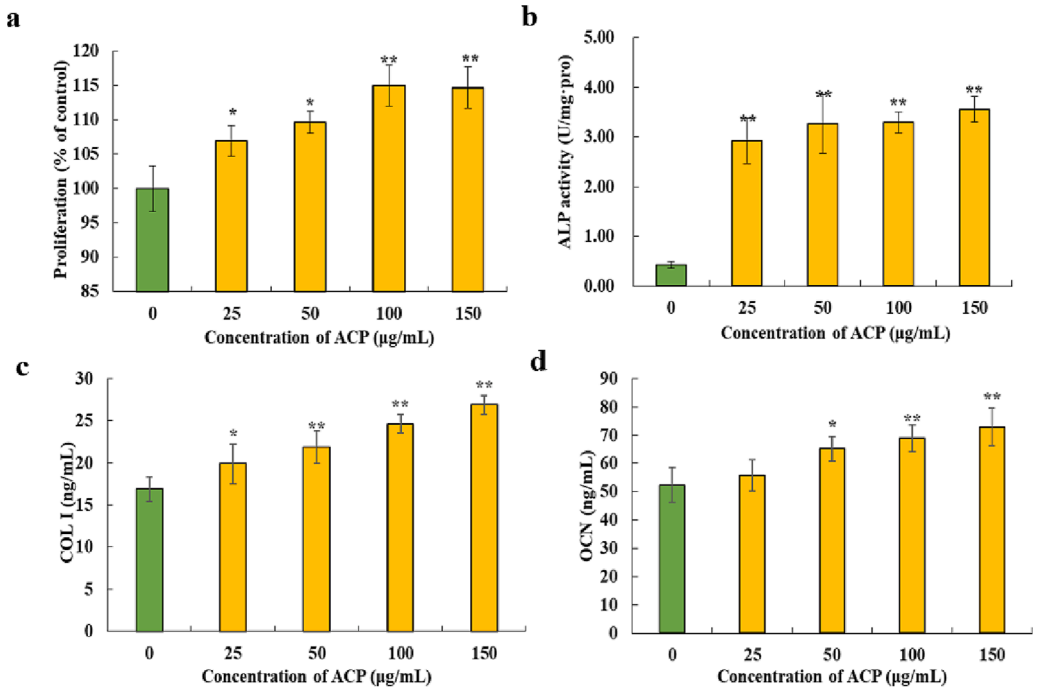

Fig. 3. Effect of ACP on proliferation (a), ALP activity (b), COL-I content (c), and OCN content (d) of pre-osteoblast MC3T3-E1. Values are expressed as means ± standard deviations (n = 3). *, the difference is significant at 0.05 level ($p < 0.05$); **, the difference is significant at 0.01 level ($p < 0.01$).

activity of ALP. ALP is a significant marker of the early differentiation of osteoblasts, and its activity reflects the degree of osteoblast differentiation [38]. Compared with the control group, the expression of ALP in the ACP groups was notably rising ($p < 0.01$), with no notable difference in the four ACP concentrations of 25 μg/mL, 50 μg/mL, 100 μg/mL, and 150 μg/mL ($p > 0.05$). (Fig. 3b). As the ACP concentration was 50 μg/mL, the ALP activity was 8.26 times that of the control group. Therefore, ACP could stimulate osteoblasts to grow and differentiate.

3.5. The effect of ACP on the COL-I and OCN content

COL-I is recognized as a representative marker protein in the middle osteoblast differentiation, which is in charge of the formation of extracellular matrix and involved in osteogenesis [39]. As seen in Fig. 3c, the ACP-treated groups showed higher COL-I than in the control group ($p < 0.01$). The COL-I level in the experimental groups showed an increasing dose-dependent effect, and no obvious difference was noted in ACP

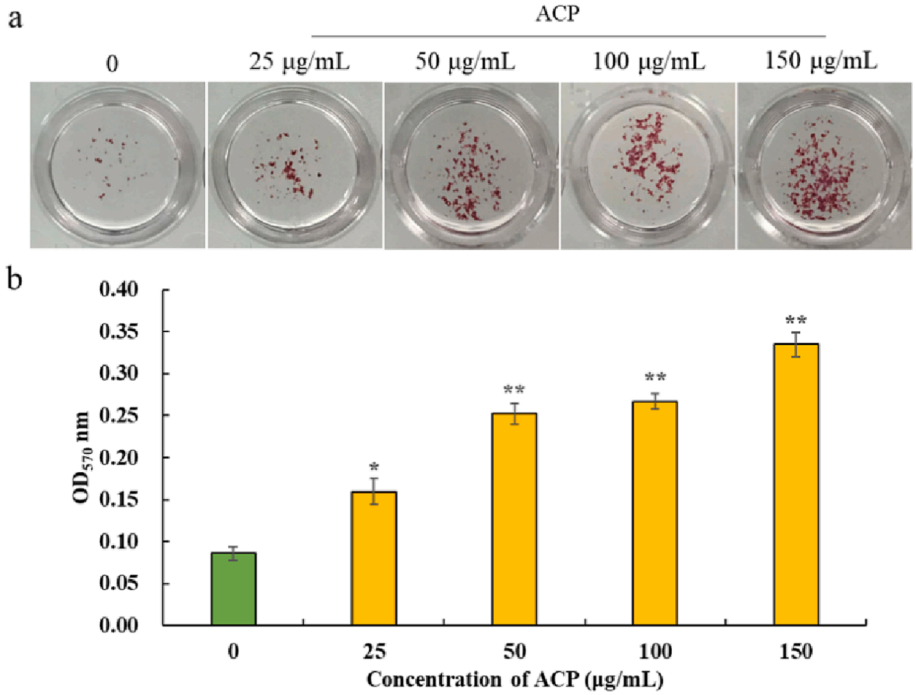

Fig. 4. Effect of ACP on mineralized nodules of pre-osteoblast MC3T3-E1. Values are shown by means ± SD (n = 3). *, the difference is significant at 0.05 level ($p < 0.05$); **, the difference is significant at 0.01 level ($p < 0.01$).

concentration between 50 μg/mL and 150 μg/mL. The result revealed that ACP could induce MC3T3-E1 cells to secret COL-I.

OCN is a specific symbol of late osteoblast differentiation [40]. It participates to regulate the deposition of bone matrix, the calcification of bone tissue and the content of bone calcium, which is the basis of mineralization [41]. As shown in Fig. 3d, ACP had a notable dose-dependent effect on OCN content. It could notably stimulate the OCN content when compared with the control group ($p < 0.01$). As a result, ACP could markedly stimulate the late differentiation and mineralization of osteoblasts.

3.6. The effect of ACP on the formation of osteoblast mineralized nodules

Mineralized bone nodules are the most intuitive manifestation of osteogenesis. Ca^{2+} could specifically combine with alizarin red S to turn red, and its absorbance is positively correlated with calcium deposition. These results could suggest the extent of mineralization of osteoblasts directly [42]. The number of calcium nodules in ACP groups increased markedly when comparing with the control group ($p < 0.01$), and it also had a notable dose-dependent effect (Fig. 4a).

The calcium nodules were dissolved in cetylpyridinium chloride solution for semi-quantitative analysis, and the results were shown in Fig. 4b. ACP could significantly induce the mineralization of MC3T3-E1 cells. Compared with the control group, the calcium nodule increased the most (289.53%) when the dose of ACP was 150 μg/mL.

4. Discussion

As one of the "Three trees" in Hainan Province, Areca nut is one of the main sources of income for>2 million farmers. However, there are few studies on Areca nut polyphenols at present. This work optimized the ultrasound-assisted extraction conditions for maximizing the yield of Areca nut seed polyphenol. Under the optimal conditions (ultrasonic power of 87 W, ethanol concentration of 65%, extraction temperature of 62℃, and extraction time of 153 min), the actual extraction yield of polyphenols was 139.62 mg/g with a small deviation (RSD = 0.18%) compared with the model prediction. The statistical analysis of high coefficients of correlation proved the effectiveness and reproducibility of the proposed model.

In vivo, osteoblasts participate in proliferation and differentiation in response to various factors. During differentiation, pre-osteoblasts express extracellular matrix proteins, such as COL-I, ALP, and OCN, then osteoblasts are mineralized [43,44]. MC3T3-EI cells are murine calvarial pre-osteoblast cell lines, which could differentiate into osteoblasts and deposit minerals *in vitro* [45]. It is a common cell model to investigate the mechanism of osteogenesis in the world. In the previous research, we set up an ovariectomized model of osteoporosis rats to explore the mechanism of action of ACP on osteoporosis by regulating gut microbiota [5]. Our main finding revealed that the ACP treatment contributed to the bone formation via modulating gut microbiota relevant to maintained Paneth cells and lysozyme. In this research, we measured the ALP activity, the extracellular matrix calcium nodules, the content of COL-I and OCN. The experimental results revealed that ACP could promote the proliferation, differentiation and mineralization of MC3T3-E1 pre-osteoblasts.

ALP is a homologous dimer glycoprotein, whose main function is to promote osteoblast maturation and mineralization. It is an early symbol of osteoblast differentiation and extracellular matrix maturation [46]. We found that ACP remarkably enhanced the content of ALP in osteoblasts, which promoted the differentiation of osteoblasts. COL-I is one of the symbols of the early differentiation of osteoblasts, accounting for 90% of the protein secreted by osteoblasts. And OCN is a marker of late differentiation of osteoblasts. It is the staple extracellular matrix non-collagen protein specifically composited by osteoblasts, which has a significant effect in maintaining the stability of mineralization of bone tissue in the late differentiation [47]. The results manifested that ACP could notably promote the protein content of COL-I and OCN in osteoblasts, and further motivated the differentiation and mineralization of osteoblasts. The formation of mineralized nodules is a sign of osteogenic differentiation and maturity and a morphological manifestation of osteogenesis. In this study, used alizarin red staining to detect the bone nodules, and the results further confirmed that ACP had the effect of promoting the mineralization of osteoblasts. Taken together, ACP could contribute to the differentiation of MC3T3-E1 cells into mature osteoblasts and mineralization. The previous study reported that the polyphenol extracted from *Hibiscus syriacus L.* had effects on osteogenic and anti-osteoporotic by enhancing calcification and ALP activity in MC3T3-E1 and MG-63 cells [48]. Patel et al. [35] revealed that the polyphenol in the roots and leaves of Sida cordifolia significantly increased the osteoblast cell proliferation, differentiation, mineralization, and mRNA expression of osteogenic genes. Moreover, our previous study found that ACP could downregulate 5-HT and increase bone mass by inhibiting bone resorption and inducing bone formation [49]. Therefore, the effect of ACP on bone formation in this paper is consistent with the above results. The results of this work could offer ideas for improving the added value of Areca nut polyphenol.

5. Conclusions

In this work, RSM was successfully used for optimizing the extraction process of ACP. Four-factor and three-level design were adopted, then the optimum parameters were obtained by RSM optimization (ultrasonic power 87 W, ethanol concentration 65%, extraction temperature 62℃, extraction time 153 min). On the optimum conditions, the actual yield of ACP was up to 139.62 mg/g which was in fine conformity with the predicted values of the model, and the error was small (RSD = 0.18%). The findings showed that the model fitted well and the predicted ACP yield data had good accuracy. In addition, results from our work indicated that ACP could induce the proliferation, differentiation and mineralization of MC3T3-E1 pre-osteoblasts. This work could provide basic data for optimizing the extraction process of ACP. And ACP has the potential to be an effective ingredient in the treatment of osteoporosis.

CRediT authorship contribution statement

Ying Sun: Project administration, Methodology, Software, Writing – original draft. **Jinfeng Lu:** Project administration, Visualization, Writing – review & editing. **Jiaqi Li:** Writing – review & editing. **Peng Li:** Formal analysis, Investigation. **Meihui Zhao:** Supervision, Writing – review & editing. **Guanghua Xia:** Conceptualization, Investigation, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ultsonch.2023.106511.

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