Orthogonal test design for optimization of the extraction of essential oil from *Asarum heterotropoides* var. *Mandshuricum* and evaluation of its antibacterial activity against periodontal pathogens

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Abstract

The optimum extraction conditions of essential oil from *Asarum heterotropoides* var. *Mandshuricum* applied by an orthogonal $L_9(3^3)$ test were a water-to-raw material ratio of 17, a particle size of $D_{95} \leq 3.8$ mm and an extraction time of 2 h. In ingredient analysis, gas chromatography/mass spectrometry analysis identified the main components of essential oil as methyl eugenol (45.95%), safrole (17.48%) and 3,5-dimethoxytoluene (10.30%) orderly. In vitro, the minimum inhibitory concentration and minimum bactericidal concentration of essential oil were 0.01 and 0.02% against *F. nucleatum*, 0.04 and 0.08% against *P. intermedia*, and 0.005 and 0.005% against *P. gingivalis*. In vivo, administration of essential oil significantly suppressed alveolar bone resorption induced by *F. nucleatum*, with bone levels remaining comparable to non-infected mice. These results of antibacterial activity of essential oil in vitro and in vivo show the inhibition of periodontal pathogens and therapy of alveolar bone resorption in mice, suggesting that its essential oil could be a potential natural therapeutic agent for treatment of periodontitis in human beings.

Keywords *Asarum heterotropoides* var. *Mandshuricum* · Orthogonal experimental design · Essential oil · Antibacterial activity · Periodontal pathogens

Abbreviations

GC/MS Gas chromatography/mass spectrometry
F. nucleatum *Fusobacterium nucleatum*
P. intermedia *Prevotella intermedia*

P. gingivalis *Porphyromonas gingivalis*
CMC Carboxymethylcellulose
SPF Specific pathogen free
CEJ Cemento enamel junction
ABC Alveolar bone crest
GAM Gifu anaerobic medium
FEMA Flavor and Extract Manufacturers' Association

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Introduction

Periodontitis is a chronic infection-induced inflammatory disease characterized by the destruction of the supporting structures of the teeth, which is the most important cause of tooth loss among adults (De Pablo et al. 2009). Chronic periodontitis is initiated by an accumulation of Gram-negative, anaerobic bacteria in subgingival sites, and is particularly associated with *F. nucleatum* (Han et al. 2000), *P. intermedia* (Malm et al. 2012) and *P. gingivalis* (Nakatsuka et al. 2014). Furthermore, chronic periodontitis is considered to be a potential risk factor

for systemic diseases such as pneumonia (Azarpazhooh and Leake 2006), cardiovascular disease (Amar et al. 2003), diabetes (Allen et al. 2011), rheumatoid arthritis (Mercado et al. 2003), osteoporosis (Megson et al. 2010) and complications during pregnancy (Nabet et al. 2010).

The most common treatment for periodontitis is the application of multiple doses of antibiotics in conjunction with mechanical therapy such as scaling and root planing (Boberts and Moncla 1988). However, excessive use of antibiotics can result in an increase in antibiotic-resistant bacteria (Ge et al. 2011), alterations in the oral and intestinal flora and undesirable side effects such as vomiting, diarrhea and tooth staining (Slots and Ting 2010). Therefore, alternative products that are safe, effective and economical are urgently needed.

Essential oils are aromatic oily liquids obtained from plant materials. The use of essential oils as functional antibacterial agents is increasing in dentistry (Matsumura et al. 2000). Various studies contributed to the evidence that essential oils may be useful in the maintenance of oral hygiene and the prevention of dental disease (Grosso et al. 2002). A number of studies have shown that the oils in some plants exhibit growth-inhibiting and bactericidal effects on oral pathogens (Takarada et al. 2004). Essential oils also inhibit co-aggregation between early colonizers and late colonizers, e.g., Gram-negative anaerobic periodonto-pathogens (Ouhayoun 2003).

The essential oil of Chinese wild ginger (*Asarum heterotropoides* var. *Mandshuricum*) has been used in traditional Chinese medicine as an analgesic, anti-tussive, and anti-allergic agent (Hashimoto et al. 1994). Previous studies have shown that the oil from Chinese wild ginger exhibits anti-viral activity (Deng et al. 2004), antioxidant activity (Chen et al. 2004), anti-pest activity (Perumalsamy et al. 2010) and anti-phytopathogenic activity (Dan et al. 2010). The chemical components in the oil have been analyzed and a total of 64 components have been identified, among which methyl eugenol was found to be the most abundant (Liu and Liu 2010). The 'green' consumerism has led to an increased scientific interest in these natural antibacterial substances. Currently, there is no published information about the antibacterial activity of the essential oil from Chinese wild ginger against oral pathogens. Therefore, we used an orthogonal experimental design to optimize the extraction conditions for obtaining a high yield of essential oil and then evaluated the antibacterial activity of the essential oil against three periodontal pathogens including *F. nucleatum*, *P. intermedia* and *P. gingivalis*. We also further investigated the inhibitory effect of the essential oil on *F. nucleatum*-induced alveolar bone resorption in mice.

Materials and methods

Plant materials

Dried roots of Chinese wild ginger were collected from Xinbin Manchus (Fushun, China) authenticated by PhD Shumei Zhang (Dalian Natural History Museum, Dalian, China). The plants were sampled and harvested in October 2015 and dried at room temperature (20–25 °C) over a period of 14 days. A voucher specimen (No. 262) was deposited in the herbarium of the Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences, China. The plant material was ground for 5 s in a laboratory pulverizer (Model WK-800A, Fuerkang Company, Qingzhou, China) at 10 s intervals with passage through standard sieves of 20, 40, 60, 80, and 100 mesh (ISO3310-1:1990, R40/3, Kangdaxin Machinery Company, Xinxiang, China).

Extraction of essential oil

Ground wild ginger powder (20 g) was placed in a round bottom flask (2 L), which was heated in a heating jacket (Model BXHW, Yuhua Company, Gongyi, China) at 150 °C and extracted testing the variables of the ratio of water-to-raw material, particle size, extraction time and soaking time. The oil was stored in sealed glass vials at 4 °C prior to analysis. The yield of oil was calculated using the following equation:

$$\text{Yield of oil (\%)} = \frac{\text{weight of oil}}{\text{weight of ground materials (20 g)}} \times 100\%.$$

Optimization of oil extraction

An orthogonal $L_9(3^3)$ test design was used for optimization of extraction conditions. Nine extractions were carried out using ratios of water-to-raw material of 14, 17, and 20, particle sizes of $D_{95} \leq 3.8$ mm, ≤ 2.5 mm and ≤ 1.8 mm (equivalent to 95% of the total amount passing through the standard sieves of No. 40, 60 and 80, respectively) and extraction times of 2, 2.5 and 3 h using single-factor

Table 1 Factors and levels for the orthogonal test

Variable	Level		
	1	2	3
A. Ratio of water-to-raw material	14	17	20
B. Extraction time (h)	2	2.5	3
C. Particle size (mm)	$D_{95} \leq 3.8$	$D_{95} \leq 2.5$	$D_{95} \leq 1.8$

tests. Table 1 shows the experimental conditions for the extraction of oil from Chinese wild ginger.

GC/MS analysis

GC–MS analysis was performed on an Agilent 6890N Gas Chromatograph (Hewlett–Packard, Palo Alto, California) coupled with a 5973 N mass spectrometer (Hewlett–Packard, Palo Alto, California) equipped with a HP-5MS capillary column (30 m, 0.25 mm i.d., 0.25 µm film thickness; J&W Scientific, San Francisco, CA). The oven temperature was held at 50 °C for 2 min, raised to 300 °C at a rate of 20 °C/min and then held at 300 °C for 5 min. The source temperature was 300 °C and the transfer line was set at 280 °C. Helium was used as the carrier gas at a flow rate of 1 mL/min. The injection volume was 1 µL, the split ratio was 1:50 and the acquisition mass range was 30–500 m/z. All mass spectra were acquired in electron-impact (EI) mode with an ionization voltage of 70 eV. Compounds were identified by comparison of their retention indices and mass spectra with those found in the literature (Palombo 2009) and the NIST Mass Spectral Library (2005 Version).

In vitro antibacterial tests

F. nucleatum (JCM 11024), *P. intermedia* (JCM 12248) and *P. gingivalis* (JCM 12257) were obtained from the Japanese Collection of Microorganisms (Ibaraki, Japan). The liquid medium used in this study was enriched Gifu anaerobic medium broth while the solid media 14 (0.24% Lab-lemco powder, 1% proteose peptone No. 3, 0.5% yeast extract, 0.4% Na₂HPO₄, 0.15% glucose, 0.05% soluble starch, 0.02% L-cystine, 0.05% L-cysteine·HCl·H₂O, 1.5% agar, 5% (v/v) horse blood) and 469 (4% trypticase soy agar, 0.0005% hemin, 0.00005% menadione, 5% (v/v) horse blood) used in this study were prepared following the instructions of the Japanese Collection of Microorganisms (Du et al. 2011). Bacteria were cultured at 37 °C in an anaerobic chamber (UniTech Bioscience, Guangzhou, China) with 85% N₂, 10% H₂ and 5% CO₂. Before use, bacteria were re-sown over 24 h on the same maintenance culture medium to obtain young strains in full growth. The minimum inhibitory concentrations of essential oil against the three bacteria were tested by broth double dilutions. The oil sample was dispersed as an emulsion in Gifu anaerobic medium broth using Tween 80 dissolved in 95% ethanol solution. To avoid any influence on bacterial growth, the proportion of Tween 80 was 1% (v/v) (Maeda et al. 2003). Serial dilutions (0.16–0.005% for *F. nucleatum*, 0.16–0.01% for *P. intermedia* and 0.16–0.0025% for *P. gingivalis*) of essential oil were prepared in Gifu anaerobic medium broth (4 mL), followed by the addition of 30 µL *F. nucleatum*, *P. intermedia* and *P. gingivalis*

suspensions (10⁸ CFU/mL), respectively, and cultured under anaerobic conditions. Tween 80 (1%) dissolved in 95% ethanol was used as the negative control, serial dilutions (40–120 µg/mL) of tinidazole was used as the positive control and GAM broth alone was used as the blank control.

The antibacterial activity of the oil was evaluated by determining the minimum inhibitory concentration and the minimum bacterial concentration according to the method of Koba et al. (2011). The minimum inhibitory concentration was determined as the lowest concentration of the test active ingredient that resulted in a complete inhibition of visible growth of the microorganism. After the measurement of minimum inhibitory concentration, 100 µL aliquots of cultures were taken from tubes showing no bacterial growth, inoculated onto blood agar plates (*F. nucleatum* and *P. intermedia* on the solid media 14, *P. gingivalis* on media 469), and cultured for 1 week under anaerobic conditions. The concentration at which no bacterial growth was observed was defined as the minimum bacterial concentration. All tests were carried out in triplicate.

Mouse periodontitis model and measurement of alveolar bone resorption

Eighteen male SPF Kunming mice, each weighing 18–20 g, were purchased from the Experimental Animal Centre at Dalian Medical University (Dalian, China). The animals were kept under a 12-h light/dark cycle with controlled humidity (60 ± 5%) and temperature (22 ± 2 °C), and were allowed free access to food and water. They were acclimatized for at least 5 days before the experiment was initiated.

As shown in Fig. 1, all mice were given 0.1% tinidazole solution, ad libitum, for 10 days to reduce their commensal oral flora, followed by a 3 days antibiotic-free period before being challenged with *F. nucleatum*. To study the inhibition

Fig. 1 Experimental procedure. Mice were divided into three groups ($n=6$ per group). Group A were administered with sterile water alone, with no infection; group B was orally infected with *F. nucleatum* and then orally administered with CMC; group C was infected with *F. nucleatum* and then orally administered with essential oil in CMC. CMC carboxymethylcellulose

of alveolar bone loss, mice were divided randomly into three groups of six mice (Group A, Group B and Group C). Mice in group A were administered with sterile water alone, with no infection. Mice in group B and C both were firstly infected with 0.1 mL *F. nucleatum* (1×10^9 colony-forming units) suspended in 2% CMC by gavage three times at 48 h intervals. Subsequently, mice in group C were given 2% Chinese wild ginger oil 14 times at 3 days intervals, while the mice in group B only received 2% CMC. At the end of the experimental period, all mice were sacrificed by cervical dislocation. All animal experiments were performed in compliance with the recommendations of the Local Institutional Animal Care and National Act on the use of experimental animals (Beijing, China).

After being excised, both maxillary halves were then defleshed and stained with aqueous methylene blue (1%) to differentiate bone from teeth. The bone loss was analyzed using an anatomical lens (30 $\times$) to measure the distance between the CEJ and the ABC, based on the method described by Nakatsuka et al.

Statistical analysis

Data were analyzed with the one-way analysis of variance (ANOVA) followed by Tukey's *t* test using GraphPad Prism 5.03 Software (GraphPad Software Inc., San Diego, CA, USA). The results were expressed as the mean $\pm$ SD. Differences between groups were considered to be statistically significant at the $P < 0.05$ level.

Results and discussion

Effect of the ratio of water-to-raw material on the yield of oil from Chinese wild ginger

The yield (%) of essential oil was affected by the ratio of water-to-raw material (Fig. 2a), when the other three factors were fixed at a particle size of $D_{95} \leq 2.5$ mm, an extraction duration of 2 h and a soaking duration of 0 h. The yield of oil increased as the ratio of water-to-raw material increased and reached its peak value ($1.85 \pm 0.02\%$) when the ratio of water-to-raw material was 17 and then it dropped as the ratio dropped from 17 to 23.

Effect of particle size on the yield of oil from Chinese wild ginger

The yield (%) of oil (Fig. 2b) was affected by particle sizes $D_{95} \leq 8.3$ mm, ≤ 3.8 mm, ≤ 2.5 mm, ≤ 1.8 mm, and ≤ 1.5 mm (equivalent to 95% of the total amount passing through the standard sieves of No. 20, 40, 60, 80, and 100, respectively) when the other three factors were fixed at

a ratio of water-to-raw material of 17, extraction time of 2 h and soaking time of 0 h. The yield of essential oil was enhanced to the maximum value ($1.98 \pm 0.02\%$) when the particle size was $D_{95} \leq 3.8$ mm. The extraction yield of oil started to decrease sharply with particle sizes reduced to $D_{95} \leq 2.5$ mm. It is suggested that particle size should be taken into consideration when using a heat-reflux extraction system for extraction of Chinese wild ginger as excessively small particle sizes do not favor the extraction of oil.

Effect of extraction duration on yield of essential oil from Chinese wild ginger

The yield (%) of oil was affected by extraction duration (Fig. 2c), when the other three factors were fixed at a ratio of water-to-raw material of 17, a particle size of $D_{95} \leq 2.5$ mm and a soaking time of 0 h. The extraction yield increased gradually with increased extraction time, and the maximum yield ($1.89 \pm 0.01\%$) was obtained when the extraction duration was 2.5 h. No further increase in extraction yield was observed when the extraction duration was extended beyond 2.5 h.

Effect of soaking duration on extraction yield of oil from Chinese wild ginger

The yield (%) of oil was unaffected by soaking duration (Fig. 2d), when the other three factors were fixed at a ratio of water-to-raw material of 17, a particle size of $D_{95} \leq 2.5$ mm and an extraction time of 2 h. The extraction yield changed slightly with increasing soaking duration. This result suggests that soaking duration has no significant effect on extraction yield.

Optimization of the extraction parameters of essential oil from Chinese wild ginger

Based on the single-factor tests above, water-to-raw material ratio, particle size and extraction time appear to be the most important factors that affect the yield of essential oil from Chinese wild ginger. In the present study, these factors were examined using an orthogonal $L_9 (3^3)$ test design. The results of the orthogonal test are shown in Table 2. The results indicate that the maximum yield of essential oil was 2.05%. However, we cannot select the best extraction conditions based only on the outcomes in Table 2 and therefore a further orthogonal analysis was warranted. Therefore, the values of K , $\bar{k}$ and R values were calculated and listed in Table 2. As seen from Table 2, the influence on the extraction yield of essential oil decreased in the order: A > C > B according to the R values observed. The water-to-raw material ratio was found to be the most important determinant of yield according to the R values. The maximum yield of

Fig. 2 Effect of extraction parameters on the extraction yield of essential oil from Chinese wild ginger. **a** Ratio of water-to-raw material, **b** particle size, **c** extraction time, **d** soaking time

essential oil was obtained when the ratio of water-to-raw material, particle size and extraction time were $A_2C_1B_3$ (17, $D_{95} \leq 3.8$ mm, and 3 h), respectively. In other words, three levels of the other factors can be ignored for the extraction yield. To save time for industrialization, the optimum technology was made as follows: $A_2C_1B_1$ (17, $D_{95} \leq 3.8$ mm, and 2 h).

Major chemical components of the essential oil

Thirty-one peaks were detected by GC/MS (Fig. 3). Thirty-one compounds were identified in the oil representing (97.91%) of the detected constituents as shown in Table 3.

The major constituents detected in the oil were methyl eugenol (45.95%), safrole (17.48%), 3,5-dimethoxytoluene (10.30%), myristicin (5.55%) and eucarvon (4.51%). Methyl eugenol, as a major component in the essential oil of the Chinese wild ginger, is widely distributed in other aromatic plants which possess a wide spectrum of activities against microorganisms ranging from bacteria to fungi (Kivanç 1988). It was reported that methyl eugenol inhibited quorum sensing by interfering with the acyl homoserine lactone activity and thus inhibited the production of violacein pigment, swimming and swarming motility, exopolysaccharides production and biofilm formation in bacterial pathogens (Sybiya Vasantha Packiavathy et al. 2012). Methyl eugenol and

Table 2 Analysis of $L_9(3)^3$ test results

No.	A, ratio of water-to-raw material	B, extraction time	C, particle size	Extraction yield (%)
1	1	1	1	1.70
2	1	2	2	1.68
3	1	3	3	1.59
4	2	1	2	1.85
5	2	2	3	1.83
6	2	3	1	2.05
7	3	1	3	1.67
8	3	2	1	1.77
9	3	3	2	1.98
K_1	4.97	5.22	5.52	16.12 (T)
K_2	5.73	5.28	5.51	
K_3	5.42	5.62	5.09	
$\bar{k}_1$	1.66	1.74	1.84	
$\bar{k}_2$	1.91	1.76	1.84	
$\bar{k}_3$	1.81	1.87	1.70	
R	0.76	0.40	0.43	

K_1 represents three mixed values of extraction yield under the first level, and the same as K_2 and K_3 ; $\bar{k}_1$ represents the value obtained by dividing K_1 by the test times under the same level, the same as $\bar{k}_2$ and $\bar{k}_3$; R refers to the result of extreme analysis

estragole are common components of spices, and they were used as flavouring substance in condiments, non-alcoholic beverages and baked goods. The FEMA Expert Panel concluded that methyl eugenol is “generally recognized as safe” under conditions of intended use as flavouring substance in food (Hall and Oser 1965). Myristicin, a methylenedioxyphenyl-containing phenylpropene constituent of the leaves of

many plants in the family Umbelliferae, is a highly effective synergist of the co-occurring furanocoumarin xanthotoxin. An unsaturated side chain on the aromatic ring of myristicin by targeting the cell wall of the bacterium may be responsible for its antibacterial potential (Narasimhan and Dhake 2006). Besides, 4-terpinenol and γ -terpinene, which were reported as important antibacterial substances, were detected in the essential oil. γ -terpinene, found in all the chemotypes studied, was certified as the initial monoterpene substrate of many other compounds such as thymol. Tohidi et al. (2017) have shown that the major constituents in the thyme including thymol biosynthesized by γ -terpinene exhibited strong biological activity. Also, 4-terpinenol and γ -terpinene were identified as major components of tea tree oil which showed a strong inhibitory effect on *F. nucleatum* and *P. gingivalis* (Zhai et al. 2008). Therefore, the small amount of 4-terpinenol and γ -terpinene incorporated in the oil from Chinese wild ginger might also take part in the growth-inhibiting and bactericidal effects on *F. nucleatum* and *P. gingivalis*.

Antibacterial activity

The antibacterial activity of the oil from Chinese wild ginger was investigated in terms of minimum inhibitory concentration and minimum bacterial concentration. As shown in Table 4 and Figs. 4, 5 and 6, the three bacterial strains tested were all sensitive to essential oil from Chinese wild ginger and *P. gingivalis* was particularly affected with markedly low minimum inhibitory concentration and minimum bacterial concentration (both 0.005%). The minimum inhibitory concentrations were 0.01% against *F. nucleatum* and 0.04% against *P. intermedia*. The minimum bacterial concentration of the oil against *F. nucleatum* and *P. intermedia* were two times

Fig. 3 Total ion chromatograms of essential oil from Chinese wild ginger

Table 3 Chemical compounds found in essential oil from Chinese wild ginger (%)

Compound	RI	Formula	Molecular weight	Proportion (%)
α -Pinene	939	C ₁₀ H ₁₆	136	0.53
Camphene	954	C ₁₀ H ₁₆	136	0.16
β -Pinene	976	C ₁₀ H ₁₆	136	0.83
β -Myrcene	991	C ₁₀ H ₁₆	136	0.13
α -Phellandrene	1007	C ₁₀ H ₁₆	136	0.30
3-Carene	1011	C ₁₀ H ₁₆	136	1.14
<i>p</i> -Cymene	1025	C ₁₀ H ₁₄	134	0.04
Limonene	1029	C ₁₀ H ₁₆	136	0.10
Eucalyptol	1031	C ₁₀ H ₁₈ O	154	0.82
γ -Terpinene	1060	C ₁₀ H ₁₆	136	0.09
4-Carene	1102	C ₁₀ H ₁₆	136	0.24
Camphor	1146	C ₁₀ H ₁₆ O	152	0.20
Eucarvone	1152	C ₁₀ H ₁₄ O	150	4.51
Borneol	1171	C ₁₀ H ₁₈ O	154	1.00
Terpinene-4-ol	1177	C ₁₀ H ₁₈ O	154	0.39
α -Terpinenol	1189	C ₁₀ H ₁₈ O	154	0.52
Estragole	1198	C ₁₀ H ₁₂ O	148	0.63
2-Isopropyl-5-methyl-anisole	1222	C ₁₁ H ₁₆ O	164	0.14
3,5-Dimethoxytoluene	1268	C ₉ H ₁₂ O ₂	152	10.30
Safrole	1283	C ₁₀ H ₁₀ O ₂	162	17.48
β -Elemene	1387	C ₁₅ H ₂₄	204	0.09
Methyl eugenol	1405	C ₁₁ H ₁₄ O ₂	178	45.95
Cyclosativene	1492	C ₁₅ H ₂₄	204	0.31
Myristicin	1522	C ₁₁ H ₁₂ O ₃	192	7.12
<i>n</i> -Pentadecane	1500	C ₁₅ H ₃₂	212	0.95
Elemicine	1552	C ₁₂ H ₁₆ O ₃	208	2.64
α -Elemene	1623	C ₁₅ H ₂₄	204	0.18
Kakuol	1646	C ₁₀ H ₁₀ O ₄	194	0.61
Patchouli alcohol	1659	C ₁₅ H ₂₆ O	222	0.29
<i>n</i> -Hexadecanoic acid	1962	C ₁₆ H ₃₂ O ₂	256	0.15
Kaurene	2048	C ₂₀ H ₃₂	272	0.08
Total identified				97.92

RI Retention indices (RI) relative to *n*-alkanes on HP-5MS capillary column (Tohidi et al. 2017; Ghaffari et al. 2018; Senatore et al. 2013; Miyazawa et al. 2008; Bunrathep et al. 2006; Liu et al. 2011; Glamočlija et al. 2011; Tamura et al. 1993; Wu et al. 2014)

Table 4 Minimum inhibitory concentration (MIC) and minimum bacterial concentration (MBC) for essential oil from Chinese wild ginger against periodontal pathogens

Strains	Essential oil		Tinidazole	
	MIC (%)	MBC (%)	MIC (μ g/mL)	MBC (μ g/mL)
<i>F. nucleatum</i>	0.01	0.02	100	120
<i>P. intermedia</i>	0.04	0.08	80	100
<i>P. gingivalis</i>	0.005	0.005	60	100

Fig. 4 Inhibition effect of serial diluted essential oil on the growth of *Fusobacterium nucleatum* in Gifu anaerobic medium broth. **a–f** Various concentrations of essential oil in the range of 0.16–0.005%; **g** 100 μ g/mL tinidazole; **h** 1% Tween 80; **i** *F. nucleatum* only **Fig. 5** Inhibition effect of serial diluted essential oil on the growth of *Prevotella intermedia* in Gifu anaerobic medium broth. **a–e** Various concentrations of essential oil in the range of 0.16–0.01%; **f** 100 μ g/mL tinidazole. **g** 1% Tween 80; **h** *P. intermedia* only **Fig. 6** Inhibition effect of serial diluted essential oil on the growth of *Porphyromonas gingivalis* in Gifu anaerobic medium broth. **a–g** Various concentrations of essential oil in the range of 0.16–0.0025%; **h** 100 μ g/mL tinidazole, **i** 1% Tween 80, **j** *P. gingivalis* only

the minimum inhibitory concentrations. In comparison, the minimum inhibitory concentration and the minimum bacterial concentration of the oil toward *F. nucleatum* and *P. gingivalis* were much lower (10–200 times) than other essential oils previously tested from manuka, tea tree, eucalyptus, lavandula, and romarinus as reported by Takarada et al. (2004).

Effect of Chinese wild ginger oil on *F. nucleatum*-induced alveolar bone resorption in mice

Chronic periodontitis is an inflammatory disease of the supporting tissues of the teeth that is characterized by the

Fig. 7 The effects of Chinese wild ginger oil on the loss of alveolar bone caused by *Fusobacterium nucleatum*. **a** Representative morphometric images for the loss of alveolar bone in mice with different treatments. Vertical bars indicate the distances from cementum–enamel junction to alveolar bone crest that were recorded; **b** linear

measurement from the cementum–enamel junction to the alveolar bone crest. Data are presented as mean values $\pm$ SD ($n=6$). Values with different panel letters are significantly different from each other ($P<0.05$), as determined by Tukey's t test

accumulation of immune cells in gingival connective tissue, resorption of alveolar bone, and the degradation of periodontal connective tissues, which lead to increased tooth mobility and eventual tooth loss (Nakatsuka et al. 2014). Additionally, one of the most studied bacteria implicated in periodontal disease is *F. nucleatum*. It belongs to the bacteroidaceae family and is a dominant micro-organism within the periodontium (Signat et al. 2011). Therefore, *F. nucleatum*-induced resorption of alveolar bone is a widely used animal experimental model for drug development in the treatment of periodontitis. Accordingly, the results showed that mice challenged with *F. nucleatum* alone showed obvious bone loss ($P<0.05$) during the experimental period, especially the third molars, reflecting the role of *F. nucleatum* on periodontal disease. In contrast, mice given essential oil after *F. Nucleatum* inoculation showed a marked decrease ($P<0.05$) in bone loss (Fig. 7). This therapeutic effect of Chinese wild ginger oil on periodontitis in animals are not only associated with inhibiting or killing of periodontal pathogens colonized in oral cavity, but also its anti-inflammatory effects by improving adrenal cortical functions, inhibiting leukocyte migration and enhancing capillary permeability. On the basis of advances in molecular and cellular biology to periodontitis, further studies are needed to clarify the precise mechanism of action and to determine if administration of the essential oil is beneficial for patients with periodontitis.

Conclusion

In this study, the extraction process of Chinese wild ginger oil was optimized by an orthogonal test design and the essential oil showed antibacterial activity against periodontal pathogens in vitro and therapeutic effect on periodontitis induced by *F. nucleatum* in mice, which suggest that Chinese wild ginger oil could be a potential and viable antibacterial agent and the Chinese wild ginger oil could be applied to the conditions caused by other pathogens. Further studies would be carried out to elucidate systemic toxicity and antibacterial mechanisms, and define the clinical efficacy of Chinese wild ginger oil on the prevention and/or treatment of periodontitis.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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