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The ethanol extract of *Osmanthus fragrans* attenuates *Porphyromonas gingivalis* lipopolysaccharide-stimulated inflammatory effect through the nuclear factor erythroid 2-related factor-mediated antioxidant signalling pathway☆

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ABSTRACT

Background: In the present study, we explored the effect of the ethanol extract of *Osmanthus fragrans* (EOF) on the growth and collagenase activity of *Porphyromonas gingivalis* (*P. gingivalis*). We also investigated the capacity of EOF to attenuate *P. gingivalis* lipopolysaccharide (LPS)-induced inflammatory responses and the possible signalling pathway.
Methods: EOF was obtained by soaking the *O. fragrans* powder in the ethanol and concentrating the extracts under reduced pressure. Microplate dilution assays were used to determine the effect of EOF on *P. gingivalis* growth. Collagenase inhibition was detected using fluorometric and colorimetric assays. The effects of EOF on the production of the cytokines interleukin-6 (IL-6) and IL-8 were assessed using enzyme-linked immunosorbent assays (ELISAs). The oxidative stress biomarkers were assayed using commercial kits. The effects of EOF on the expression of cytoprotective enzymes and nucleoprotein nuclear factor erythroid 2-related factor (Nrf2) were tested by Western blot analysis.
Results: EOF significantly inhibited the growth of *P. gingivalis*, especially in the iron-limited culture medium. The inhibitory effect of EOF on *P. gingivalis* collagenase activity was time- and concentration-dependent. The *P. gingivalis* LPS-stimulated production of IL-6 and IL-8 was attenuated by EOF. LPS significantly induced the production of nitric oxide (NO) and malondialdehyde (MDA), and decreased the expression of superoxide dismutase (SOD) while pretreatment with EOF alleviated these effects. The presence of EOF markedly upregulated the expression levels of the cytoprotective enzymes and nucleoprotein Nrf2.
Conclusion: This study suggests that the potent Nrf2 activation capacity of *O. fragrans* may be useful in the adjunctive treatment of periodontal disease.

☆ This work should be attributed to Nanfang Hospital, Southern Medical University.

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1. Introduction

Periodontal diseases or periodontitis are multifactorial inflammatory disorders initiated by the combined effects of the accumulation of Gram-negative anaerobic bacteria and an overactive host-cell immune response. This interaction inevitably leads to the irreversible resorption and destruction of tooth-supporting tissue. It has been estimated that nearly 53.1–58.8% of adults are affected by periodontitis in China,[1] whereas the prevalence of periodontitis is much lower in the United States, where 30% of adults are affected.[2] Therefore, periodontitis, which is quite common, could result in tooth loss and systemic complications if not treated timely and properly. *Porphyromonas gingivalis* (*P. gingivalis*) has long been recognized as the key pathogens in the chronic periodontitis.[3] Periodontopathogen lipopolysaccharide (LPS), a major component of the outer membrane of Gram-negative bacteria, such as *P. gingivalis*, inhibits osteoblastic differentiation and initiates the cascade of events leading to periodontal tissue destruction.[4] LPS is considered an important virulence factor of *P. gingivalis* in the pathogenesis of periodontitis owing to its induction of various pro-inflammatory cytokines such as interleukin (IL)-1β and tumour necrosis factor (TNF)-alpha in inflammatory cells[5,6]; these cytokines have been reported to accelerate the process of osteoclast and bone resorption potently, both in vivo and in vitro.[7,8] LPS is also known as a potent stimulator of matrix metalloproteinases (MMPs), which play an essential role in periodontal tissue breakdown.[9] Therefore, the inhibition of the virulent effects of *P. gingivalis* may be a potentially effective strategy for the prevention and control of periodontal disease.

There is a growing interest in natural compounds that can be used to prevent and cure illness. Various plants such as tea, coffee, grape, and a host of others are rich in polyphenols.[12] Different groups of polyphenols may be classified according to the number of phenol rings in their chemical structure and the structural elements the rings bind.[13] They abound in our diet and contribute to the prevention of cancer, cardiovascular disease, and obesity through their antioxidant properties.[10,11] Polyphenols may also be potential therapeutic options in the pharmacological adjuvant management of periodontitis because of their well-known antimicrobial and anti-inflammatory properties.[14–16]

Osmanthus fragrans, an evergreen shrub with eye-catching foliage, is a species of Oleaceae native to Asia, particularly the south of China. Its flowers, called Kwai-fah in China, are small, sweet-scented, and occur in three different shades of yellow including pale, golden, and orange-yellow. It is displayed for aesthetic purpose and also used as an additive for tea and foods such as cake, pastry, paste, vinegar, and liqueurs. In ancient times, *O. fragrans* was used in traditional Chinese medicine to treat weakened vision, halitosis, dyspnoea, asthma, cough, toothache, stomach ache, diarrhoea, and hepatitis. The efficacy of this plant may be attributed to the high content of flavonoids and polyphenols.[17,18] Although scientific evidence of the in vivo therapeutic activity of *O. fragrans* is yet to be provided, a few studies have examined the in vitro bioactivity. For example, the flower of *O. fragrans* has been demonstrated to exhibit antioxidant capacity,[19] attenuate nitric oxide production,[20] scavenge free radicals for neuroprotection,[21] and inhibit melanogenesis.[22,23] In addition, Wang et al.[24] found that the flavonoids from *O. fragrans* possessed significant antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis*, and *Escherichia coli*. Thus, we hypothesize that *O. fragrans* is capable of exerting antimicrobial effect against *P. gingivalis* through its antioxidant property. In order to test this hypothesis, we investigated the effects of the ethanol extract of *O. fragrans* (EOF) on the growth of *P. gingivalis*, the inhibitory activities of *P. gingivalis* collagenase, and the inflammatory cytokine production by human periodontal ligament cells (*h*PDLCs). Finally, we investigated its effects on the nuclear factor erythroid 2-related factor (Nrf2)-associated antioxidant signal pathway expression by *h*PDLCs stimulated with *P. gingivalis* LPS.

2. Material and methods

2.1. Plant material and the preparation of EOF

The flowers of *O. fragrans* were collected from Hubei University of Science and Technology (Xianning City, China) in the month of September 2013. Three biological replicates of each cultivar were used in each experiment. Flower samples were oven-dried at 80 °C, spread out to dry naturally, and then ground into a fine powder. All samples were immediately placed in airtight polyethylene bags, frozen and stored at −20 °C. The powder samples were soaked in 80% ethanol for 3 h at 90 °C. The ratio of powdered flowers to ethanol was 1:40. The resulting EOF was filtered using Whatman no. 1 filter paper and concentrated under reduced pressure using a rotary evaporator.

2.2. Effect of EOF on the growth of P. gingivalis

The effect of EOF on *P. gingivalis* (ATCC 33277) growth was assessed using the protocol described by Marquis et al.[25] Two different culture media were used in a microplate dilution assay. Todd–Hewitt broth (THB) supplemented with 20 μM hemin and 0.0001% vitamin K (THB-HK) was used as a complex medium with excess iron, while mycoplasma broth base (MBB) supplemented with 10 μM hemin (MBB-H) was used as a medium with limited iron. After culturing for 24 h, a uniform baseline density for *P. gingivalis* (OD_{660} = 0.2) was obtained using spectrophotometry. Equal volumes of *P. gingivalis* suspension and EOF were mixed (at concentrations of 31.25–250 μg/ml) diluted in culture media in the wells of 96-well plates. Wells with no *P. gingivalis*, or EOF, were used as controls. Following incubation at 37 °C under anaerobic conditions for 48 h, the OD_{660} was measured using a microplate reader to evaluate the bacterial growth.

2.3. Effect of EOF on P. gingivalis collagenase activity

P. gingivalis collagenase activity was estimated using the protocol described by Santos et al.[26] The *P. gingivalis* suspension was incubated for 48 h and the supernatant was collected by centrifuging at 10,000 × *g* for 10 min at 4 °C. The assay mixtures were composed of TCNB buffer (50 mM Tris–HCl,