Anti-inflammatory, Antioxidant and Antimicrobial Effects of Artemisinin Extracts from Artemisia annua L.

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The anti-inflammatory, antioxidant, and antimicrobial properties of artemisinin derived from water, methanol, ethanol, or acetone extracts of Artemisia annua L. were evaluated. All 4 artemisinin-containing extracts had anti-inflammatory effects. Of these, the acetone extract had the greatest inhibitory effect on lipopolysaccharide-induced nitric oxide (NO), prostaglandin E\(_2\) (PGE\(_2\)), and pro-inflammatory cytokine (IL-1\(\beta\), IL-6, and IL-10) production. Antioxidant activity evaluations revealed that the ethanol extract had the highest free radical scavenging activity, (91.0±3.2%), similar to \(\alpha\)-tocopherol (99.9%). The extracts had antimicrobial activity against the periodontopathic microorganisms Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum subsp. animalis, Fusobacterium nucleatum subsp. polymorphum, and Prevotella intermedia. This study shows that Artemisia annua L. extracts contain anti-inflammatory, antioxidant, and antimicrobial substances and should be considered for use in pharmaceutical products for the treatment of dental diseases.

Key Words: Anti-inflammatory effect, Anti-microbial activity, Antioxidant activity, Artemisinin

INTRODUCTION

There has been an increase is the reevaluation of traditional medicinal plants worldwide, with extensive research on various plant species and their therapeutic properties being carried out. Traditional medicinal plant remedies have been highlighted as alternative medicines that are less likely to cause adverse side effects, unlike synthetically generated chemical substances [1]. Artemisia annua L. (Asteraceae) is an annual herb native to Asia, and has been used for many centuries in traditional Asian medicine for the treatment and prevention of fever and chills [2]. A variety of compounds have been extracted from Artemisia annua L. such as sesquiterpenoids, flavonoids, coumarins, lipids, phenolics, purines, steroids, triterpenoids, alkaliphatics, and artemisinin [3].

The main component in Artemisia annua L., artemisinin, has the formula C\(_{15}\)H\(_{22}\)O\(_{15}\) and contains a peroxide bridge (C-O-O-C) (Fig. 1). Artemisinin has been widely used for the treatment of malaria for the past two decades [4]. Additionally, artemisinin is known to have antibacterial, antifungal, antileishmanial, antioxidant, antitumor, and anti-inflammatory activity [5-7].

During inflammation, macrophages are key immune cells that regulate inflammatory responses. Inflammatory responses to pathogenic microbes rely on innate and adaptive im-

Fig. 1. The structure of artemisinin.
mune responses. Activated macrophages secrete a variety of inflammatory mediators, including interleukin (IL)-1β, IL-6, IL-10, prostaglandin E2 (PGE2) and nitric oxide (NO) [8-10]. Cytokines, such as IL-1β, IL-6 and IL-10 are soluble proteins secreted by cells of the immune system [11]. Free radicals in the form of reactive oxygen and nitrogen species are an integral part of normal physiology, and free radical reactions occur throughout the human body. Overproduction of these reactive species can occur due to oxidative stress caused by imbalances in the body’s antioxidant defense system and free radical formation [12,13]. These reactive species can interact with biomolecules, causing cellular injury and death. Scavenging of 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) radicals is the basis of the widely used DPPH radical scavenging assay [14]. Periodontal disease is a complex chronic inflammatory disorder that is one of the most prevalent microbial diseases in the world. Gram-negative anaerobic bacteria are the etiologic agents of periodontal disease. Periodontal disease and severe periodontitis can result in periodontal destruction, pain, alveolar bone resorption, and tooth loss [15]. Aggregatibacter actinomycetemcomitans is a gram-negative bacterium associated with a variety of infectious diseases such as nonperiodontal and urinary tract diseases, as well as general periodontal disease and localized juvenile and adult periodontitis [16]. Members of the genus Fusobacterium are common species of human microbiota normally associated with the oral cavity, colon, genital tract, and upper respiratory tract. Fusobacterium species thrive in gingival pockets and increase in number with the progression of periodontal disease. It is one of the characteristic species frequently isolated from infected gingival pockets indicating it is a potential periodontal pathogen [17]. Prevotella intermedia is another species that is present in generalized juvenile periodontitis and found in the gingival crevice of patients with periodontitis [18,19]. Artemisinin extracts from Artemisia annua L. has been used for the treatment of a variety of human illnesses as has been reported previously [4-7]. However, Artemisinin extracts generated with different solvents and their dental pharmaceutical potential have not been reported yet. In this study, artemisinin extracts from water, methanol, ethanol, and acetonitrile were evaluated for anti-inflammatory, antioxidant, and antimicrobial effects to determine their therapeutic potential for use in dental pharmacuticals.

METHODS

Materials

Methanol, ethanol, acetonitrile, and acetonitrile were purchased from JT Baker (Phillipsburg, NJ, USA). Artemisia annua L. was purchased from the Yakryeong Market in Seoul, South Korea. Folin-Ciocalteu phenol reagent, gallic annua chased from JT Baker (Phillipsburg, NJ, USA). An Enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D Systems (Minneapolis, MN, USA). Blood agar was obtained from Asan pharmaceutical company (Asan Pharm Co., Seoul, Korea).

Preparation of artemisinin-containing solution (ACS)

Dried Artemisia annua L. (15 g) was extracted with 80% methanol, 80% ethanol, and 80% acetonite under reflux for 24 hr, and the supernatants were filtered using Whatman filter paper (Macherey-Nagel, Germany). The filtered extracts were evaporated under reduced atmospheric pressure using a rotary evaporator (Eyela, Japan), resulting in a viscous solution. Equal volumes of Artemisia annua L. were extracted with hot water and filtered using Whatman filter paper.

The artemisinin-containing Artemisia annua L. extracts were measured by the Folin-Denis method [20]. Folin-Ciocalteu phenol reagent (750 μl) was added to 150 μl of sample and the reaction mixture was incubated for 5 min. After incubation, 600 μl of 10% Na2CO3 was added and the reaction mixture was incubated at room temperature for 1 hr. Absorbance of the reaction mixture was measured at 760 nm using a microplate reader (Tecan, Männedorf, Switzerland). Artemisinin was quantified by HPLC with a Waters® 1525 binary HPLC pump, Waters® 2797 autosampler, Waters® 2489 UV/visible detector, and ODS (250×4.6 mm, 5 μm) Hypersil C-18 column (Thermo Fisher Scientific, Waltham, MA, USA) [21]. The mobile phase consisted of a 3:7 water:acetonitrile ratio with a flow rate of 1 ml/min. The injection volume for each sample was 10 μl, and the retention time of artemisinin was approximately 15 min at 220 nm.

Cell viability assays

RAW 264.7 murine macrophage cells were acquired from the Korea Cell Bank (Seoul, Korea) and cultured in DMEM containing 10% heat-inactivated FBS, and 20 μg/ml of gentamycin at 37°C in a CO2 incubator (Thermo Fisher Scientific). Cell viability was determined after treatment with 0, 1, 5, 10, 50, and 100 μg/ml of ACS. RAW 264.7 cells (3×104 cells/well) were seeded in 24 well plates. After 24 hr of treatment, cell viability was evaluated in an MTT assay [22]. Media was replaced with 500 μl of fresh serum-free media containing 0.5 mg/ml of MTT reagent. After 1 hr of incubation at 37°C in a CO2 incubator, the MTT-containing media was removed, and the reduced formazan dye was solubilized by adding 500 μl of DMSO to each well. After mixing gently, the absorbance of the solution was measured at 590 nm with a microplate reader.

Nitrite determination

RAW 264.7 cells were plated at a density of 5×105 cells in 24 well plates with 1 ml of culture medium and incubated for 24 hr. Cells were then treated with 0, 1, 5, 10, 50, and 100 μg/ml of various ACS extracts in the presence of LPS (0.1 μg/ml), and incubated for an additional 24 hr. The quantity of nitrite generated was measured using the Griess reagent assay. Equal volumes of culture supernatant and Griess reagent were mixed and incubated for 10 min at room temperature. Absorbance was measured at 540 nm on a spectrophotometer and compared to a nitrite standard curve to determine the nitrite concentration in the tissue culture wells.
**IL-1β, 6, 10, and PGE₂ determinations**

The level of IL-1β, 6, 10, and PGE₂ in supernatants from RAW 264.7 cell cultures was determined using an ELISA kit according to the manufacturer's instructions. RAW 264.7 cells were incubated with LPS (0.1 μg/ml) in the presence of different concentrations of ACS extract for 24 hr. Supernatants were collected and stored at −80°C for later analysis. Absorbance was measured at 450 nm using a microplate reader and compared to a standard curve to determine the pro-inflammatory cytokine levels present in the supernatants.

**DPPH radical scavenging activity**

DPPH radical scavenging activity of the extracts was evaluated according to the method of Hatano and Kagawa [23]. ACS extracts were added to 100 μl of DPPH solution to a final concentration of 0.2 mM, mixed gently, and allowed to stand for 30 min. The absorbance of the mixture was measured at 517 nm with a microplate reader. α-Tocopherol was used as a positive control standard and DPPH radical scavenging activity was calculated using the following equation:

\[
\text{DPPH radical scavenging activity} = (1 - \frac{A_{\text{test}}}{A_{\text{control}}}) \times 100,
\]

where \(A_{\text{test}}\) is the absorbance of the test solution and \(A_{\text{control}}\) is the absorbance of the \(\alpha\)-tocopherol solution.

**Antimicrobial activity assays**

Antimicrobial activity was determined using the agar disc diffusion method or broth micro-dilution susceptibility assay. *A. actinomycetemcomitans* KCTC 3698 was obtained from the Korean Collection for Type Cultures (KCTC; Daejeon, Korea). *F. nucleatum* subsp. *animalis* KCOM 1325, *F. nucleatum* subsp. *polymorphum* KCOM 1232, and *P. intermedia* KCOM 1107 were obtained from the Korean Collection for Oral Microbiology (KCOM; Gwangju, Korea). The bacteria were grown anaerobically on blood agar at 37°C for 18 hr. ACS extracts were applied to sterilized paper disks (10 mm, 100 μl/disc) and placed on the inoculated agar surface. After preincubation at room temperature for 1 hr, the plates were incubated at 37°C for 18 hr. Fresh media on paper discs was used as a control. A broth micro-dilution susceptibility assay was also conducted in accordance with NCCLS 2009 [24] guidelines for minimum inhibitory concentration (MIC) determinations.

**Statistical analysis**

All assays were carried out in triplicate, and the values presented are the average of 3 replicates. Statistical analyses of the differences between samples were carried out by one-way analysis of variance (ANOVA), followed by post hoc multiple comparisons with Duncan’s test and student’s t-test with the PASW (Predictive analytics software) statistics package for Windows program. Differences were considered significant if the p-value was less than 0.01.

**RESULTS**

**Artemisinin-containing solution (ACS) content**

The ACS content was determined for 1000 μg/ml of each of the *Artemisia annua* L. extracts and the results are shown in Table 1. The ACS values for water, methanol, ethanol, and acetone were 41.0±1.0, 21.5±0.7, 17.0±0.4, and 16.9±1.0 μg GAE/g, respectively.

![Fig. 2. HPLC chromatography peak area against the concentration. HPLC chromatogram showing peaks of artemisinin-containing solution (ACS). A: Water extract, B: methanol extract, C: ethanol extract, D: acetone extract. Examples of HPLC traces with the artemisinin peak labelled (*)](image)

**Table 1. Total artemisinin-containing solution (ACS) of Artemisia annua L. extracted by 4 solvents (μg GAE/g)***

| Concentration (μg/ml) | Total ACS |
|----------------------|-----------|
|                      | Water     | Methanol | Ethanol | Acetone |
| 1000                 | 41.0±1.0  | 21.5±0.7 | 17.0±0.4| 16.9±1.0|

*GAE, gallic acid equivalents, †All measurements were done triplicate, and values are average of 3 replicates. ‡Means the different superscripts within the same row are significantly different at p<0.01.
16.9±1.0 μg GAE/ml, respectively. The highest ACS content was detected in the water extract.

**Determination of artemisinin from Artemisia annua L.**

Using the HPLC conditions described above, HPLC results are shown in Fig. 2 with an artemisinin peak retention time of 6.1~6.5 min. The artemisinin content of each extract ranged from 81~221 μg/ml in water, methanol, ethanol, and acetone extracts. The highest artemisinin content was detected in the acetone extract (Table 2).

**Cytotoxicity**

The cytotoxic effects of the different ACS preparations were evaluated in RAW 264.7 cells with an MTT assay. Cells were treated for 24 hr with 0, 1, 5, 10, 50, and 100 μg/ml of each ACS preparation. The control group was treated with media. As shown in Fig. 3, all ACS preparations had no cytotoxicity.

**Generation of NO and PGE$_2$**

The inhibitory effects of the ACS preparations on NO and PGE$_2$ production in LPS-treated RAW 264.7 cells was determined. Cells were treated for 24 hr with 0, 1, 5, 10, 50, and 100 μg/ml of each ACS preparation and LPS (0.1 μg/ml). As shown in Fig. 4, the ACS preparations inhibited the production of NO and PGE$_2$ inflammatory mediators. The ACS acetone extract had the highest anti-inflammatory activity relative to the other extracts. The NO production level of RAW 264.7 cells treated with 100 μg/ml of the ACS acetone extract was inhibited by 83.3% relative to control cells. ACS ethanol and methanol extracts (100 μg/ml) had the highest inhibitory effects on PGE$_2$ production (83.3% and 78.4% inhibition, respectively).

**Effect on pro-inflammatory cytokines**

The ACS preparations were evaluated to determine their effect on the production of IL-1β, IL-6, and IL-10 in LPS-stimulated RAW 264.7 cells. As shown in Fig. 5, consistent with the observations made previously in regard to IL-1β, IL-6, IL-10, and TNF-α production, all of the pro-inflammatory cytokines evaluated were suppressed by all of the ACS preparations with the 4 solvents used. The ACS acetone extract had the highest anti-inflammatory activity compared to the other extracts. ACS acetone extract (100 μg/ml) treatment of LPS-stimulated RAW 264.7 cells had the highest inhibitory effect on the various cytokines, inhibiting production of IL-1β by 61.0%, IL-6 by 45.1%, and IL-10 by 73.0%. These results demonstrate that the ACS acetone extract has anti-inflammatory effects by inhibition of various inflammatory mediators, including pro-inflammatory cytokines.

**Table 2. Artemisinin contents of Artemisia annua L. extracted by 4 solvents (μg/mg)**

| Solvent       | Water | Methanol | Ethanol | Acetone |
|---------------|-------|----------|---------|---------|
| Artemisinin content | 81    | 171      | 154     | 211     |

Fig. 3. The cell viability of RAW 264.7 cells with the artemisinin at different concentrations. Cells were treated for 24 hr with 0, 1, 5, 10, 50 or 100 μg/ml of artemisinin-containing solution (ACS). The normal group was treated with media only. The results are expressed as the mean±SD from three independent experiments. *p<0.01 indicates significant differences from the cells.

Fig. 4. Suppression of LPS-induced NO and PGE$_2$ production in RAW 264.7 cells by the artemisinin. Cells were treated for 18 hr with 0, 1, 5, 10, 50, or 100 μg/ml of artemisinin-containing solution (ACS) in the presence of 0.1 μg/ml LPS. The results are expressed as the mean±SEM from three independent experiments. *p<0.01 indicates significant differences from the LPS treated cells. A: NO, B: PGE$_2$. 
Functional Activities of Artemisia annua L.

Fig. 5. Suppression of LPS-induced cytokine production (IL-1β and IL-10) in RAW264.7 cells by the artemisinin. RAW 264.7 cells were treated for 18 hr with 0, 1, 5, 10, 50 or 100 μg/ml of artemisinin-containing solution (ACS) in the presence of 0.1 μg/ml LPS. The results are expressed as the mean±SE from three independent experiments. *p<0.01 indicates significant differences from the LPS treated cells. A: IL-1β, B: IL-10.

Table 3. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of artemisinin-containing solution (ACS) (unit %)

| Concentration (mg/ml) | α -tocopherol | ACS | Water | Methanol | Ethanol | Acetone |
|-----------------------|---------------|-----|-------|----------|---------|---------|
| 0.3                   | 91.5±0.4      | 50.4±7.1 | 36.6±4.6 | 31.4±2.3 | 15.4±1.6 |
| 0.6                   | 91.6±0.6      | 90.6±1.1 | 70.5±2.8 | 57.0±1.0 | 33.3±1.3 |
| 1.3                   | 91.7±0.7      | 91.1±0.2 | 80.1±1.0 | 71.0±0.5 | 68.8±5.3 |
| 2.5                   | 91.7±0.8      | 90.5±0.1 | 83.1±1.1 | 76.4±3.5 | 88.7±0.5 |
| 5.0                   | 91.1±2.8      | 90.0±0.3 | 83.4±2.1 | 79.2±3.4 | 87.1±0.1 |
| 10.0                  | 92.6±0.4      | 91.0±3.2 | 85.1±9.7 | 82.3±3.9 | 90.7±0.4 |

All measurements were done triplicate, and values are average of 3 replicates. Means the different superscripts within the same row are significantly different at p<0.01.

Table 4. Comparison of antimicrobial activity of artemisinin-containing solution (ACS) (unit: mm)

| Microorganism                      | ACS (14 mg/ml) | Water | Methanol | Ethanol | Acetone |
|------------------------------------|----------------|-------|----------|---------|---------|
| A. actinomycetemcomitans           | 12.3±0.6       | ND    | ND       | ND      | ND      |
| F. nucleatum subsp. animalis       | ND             | ND    | ND       | ND      | ND      |
| F. nucleatum subsp. polymorphum    | 12.3±0.6       | 12.6±0.6 | 11.3±0.6 | 13.3±0.6 |

Inhibition zones including the diameter of the paper disc (10 mm). ND, not detected. All measurements were done triplicate, and values are average of 3 replicates.

DPPH radical scavenging activity

DPPH free radical scavenging activity was used to evaluate the antioxidant activity of the ACS extracts. As shown in Table 3, DPPH radical scavenging activity of the various ACS preparations significantly increased with increasing extract concentration. DPPH radical scavenging activities in water, methanol, ethanol, and acetone extracts (10.0 mg/ml) was 91.0, 85.1, 82.3, and 90.74%, respectively. The highest DPPH radical scavenging activity was observed with the ACS water extract.

Antimicrobial activity

Antibacterial activity of the ACS preparations was assayed with the disc diffusion assay and MIC assay. The ACS concentrations used for the disc diffusion assay were 14 mg/ml. The extracts demonstrated antibacterial activity against A. actinomycetemcomitans, F. nucleatum subsp. animalis, F. nucleatum subsp. polymorphum, and P. intermedia. The methanol, ethanol, and acetone ACS preparations inhibited P. intermedia. The water and acetone ACS preparations inhibited A. actinomycetemcomitans and F. nucleatum subsp. animalis, respectively (Table 4). The MIC for the ACS preparations ranged from 7 to 14 mg/ml (Table 5).

DISCUSSION

The anti-inflammatory, antioxidant, and antimicrobial effects of artemisinin extracts generated from Artemisia an-
nua L. using four different solvents were evaluated. The results demonstrated that artemisinin has anti-inflammatory, antioxidant, and antimicrobial activities against periodontopathic bacteria.

Inflammation is a complex biological response of tissues to harmful stimuli, such as pathogens, damaged cells, or irritants. Excessive or prolonged inflammation can lead to tissue destruction. Recent evidence shows that prolonged infection and inflammation at a local site, such as the periodontium, can have systemic implications, influencing cardiovascular disease, diabetes, and respiratory ailments.

With respect to inflammation in the oral cavity, the prevention and treatment of gingivitis and periodontitis are beneficial to maintaining a healthy dental state, which, in turn, influences a healthy body [25]. A variety of oral products with natural anti-inflammatory activity has used to relieve pain. These include products from food or herbs and plants known to reduce the symptoms of localized tissue reactions to irritation, injury, or infection. There are different types of natural anti-inflammatory agents, such as peppermint, Brazil cress, and oil of cloves that can be used to relieve pain associated with toothaches (http://www.ehow.com/about_5454048_natural-anti-inflammatory-tooth-pain.html).

Until now, compounds such as chlorhexidine, fluoride compounds, xylitol, and sorbitol have been extensively used to inhibit bacterial growth in dental caries in enamel, pulp, and periapical diseases [26]. Discoloration of teeth and prostheses and burning sensations in taste, and oral mucosa are caused by chlorhexidine [27]. Excessive fluoride ingestion may cause abdominal pain and cancer [27], and xylitol and sorbitol have disadvantages and are not cost effective.

In this study, the Korean medicinal plant Artemisia annua L. was evaluated for its anti-inflammatory, antioxidant, and antimicrobial activities to determine its potential use for the treatment of dental problems. Phenolic compounds have antioxidant scavenging capabilities due to their hydroxyl groups as measured using the Folin–Denis method [20], and are considered to be important constituents of Artemisia annua L. The current study shows that water extracted preparations had a higher ACS concentration than observed with the methanol, ethanol, and acetone extracts. However, the artemisinin concentration was highest in the acetone extract.

Pro-inflammatory cytokines and mediators regulate the functional activities of immune cells and are involved in the pathogenesis of inflammatory diseases, including septic shock, hemorrhagic shock, multiple sclerosis, rheumatoid arthritis, ulcerative colitis, and atherosclerosis [28–33]. Therefore drugs that suppress or inhibit the expression of these inflammation-associated factors are promising therapeutic approaches for the prevention of pathological inflammatory reactions and diseases. The results of this study show that inflammatory cytokine expression by LPS-stimulated RAW 264.7 cells was reduced by treatment with the various ACS samples. The ACS acetone extract had the greatest inhibitory effect on LPS-induced NO, PGE2, and cytokine production when compared to the water, methanol, and ethanol extracts.

Oxygen radicals have been shown to be involved in various diseases, including cancer, diabetes, muscular dystrophy, cataracts, cardiovascular disease, and aging [34,35]. Dietary antioxidants can improve cellular defenses and inhibit oxidative damage to cellular components [12]. In this study, the ACS water extract had the greatest antioxidant activity of all of the extracts tested.

Previous studies have shown that Artemisia annua L. extracts have antimicrobial activity against diverse pathogens, including Bacillus subtilis, Candida krusei, Enterococcus hirae, Enterococcus faecalis, Escherichia coli, Haemophilus influenzae, Pseudomonas aeruginosa, Saccharomyces cerevisiae, and Staphylococcus aureus [36,37]. However, the antimicrobial activity of this plant has not been evaluated against bacteria that cause periodontopathic disease. In this study, ACS preparations were shown to have antimicrobial effects on the growth of A. actinomycetemcomitans, F. nucleatum subsp. animalis, F. nucleatum subsp. polymorphum, and P. intermedia, which all cause periodontal diseases. According to previous studies, polyphenol-containing natural product extracts have antimicrobial effects against A. actinomycetemcomitans [38,39]. This study is consistent with a previous study that reported ACS water extracts had antimicrobial activity against A. actinomycetemcomitans. The ACS methanol, ethanol, and acetone preparations had antibacterial activity against F. nucleatum subsp. animalis, F. nucleatum subsp. polymorphum, and P. intermedia.

This is the first report demonstrating the antimicrobial activity of artemisinin against periodontopathic microorganisms.

While natural extracts are generally not considered to be sufficiently potent for the treatment of clinical infections, the results of the present study are promising. The findings that came out of this work on the therapeutic potential of artemisinin support carrying out more research to characterize the pharmacological effects of other natural products and may be useful for the development of therapeutic interventions for a variety of dental diseases.

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