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The efficacy of hesperidin for treatment of acute otitis media

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ABSTRACT

Objectives: In this experimental study, the effect of hesperidin on the treatment of acute otitis media (AOM) was investigated in an AOM-induced rat model.

Methods: In total, 35 rats were randomly divided into the following five groups (n = 7): group 1 (control), group 2 (AOM with no treatment), group 3 (AOM + antibiotic), group 4 (AOM + hesperidin), and group 5 (AOM + hesperidin + antibiotic). On day 14, group 3,4 and 5 rats were given antibiotic and hesperidin via gavages, respectively. Histopathological and immunological analyses were performed and the results analyzed.

Results: Serum levels of TNF-α, IL-4, IL-6 and IL-1β were significantly decreased in the hesperidin- and antibiotic-treated groups compared to the AOM group. The AOM + antibiotic and AOM + hesperidin groups demonstrated reduced histological damage compared to the AOM group. Between the AOM + antibiotic and AOM + hesperidin groups, significant differences in tympanic membrane thickness(ThicTM), inflammation(Inf), and sclerosis(Sc) values were observed. However, no difference in epithelial damage(DamEpith), was seen between the two groups. There was a significant difference in the AOM + antibiotic and AOM + antibiotic + hesperidin groups compared to AOM group ($P < 0.001$).

Conclusions: In this study, we observed that both antibiotic and hesperidin treatment reduced AOM symptoms in an AOM-induced rat model. The values in AOM + antibiotic + hesperidin group were markedly lower than those of the other groups. From our results, we propose that hesperidin, in combination with antibiotics, may provide a successful alternative treatment for AOM compared with antibiotics used alone.

1. Introduction

Acute otitis media (AOM) is a major condition that effects all age groups and is related to inflammation of the middle ear cavity (MEC) and upper respiratory tract. The mechanisms of AOM in the MEC that predispose an individual to the development of inflammation are partially known [1,2]. MEC inflammation is frequently elicited by viral infections and characterized by redness and bulging of the tympanic membrane. Human rhinovirus, influenza viruses, respiratory syncytial virus, adenovirus, and enterovirus are the five most common AOM-associated viruses. Bacteria can also contribute to AOM, with the most common being *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*,

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Streptococcus pyogenes, and *Staphylococcus aureus*. AOM can reduce quality of life by causing pain, hearing loss, headaches, and cognitive impairment [3,4]. Impaired function of the middle ear mucosa and subsequent stasis of infected secretions is a key contributor to the pathophysiology of AOM and is associated with local inflammation [4,5].

One of the challenges in treating AOM is that the pathogens can be resistant to commonly used antibiotics [6]. In addition to conventional therapies, several AOM patients use supplementary and alternative therapies, especially herbal preparations. One such herbal preparation is hesperidin, a flavanone glycoside found in citrus fruits and in the inner layer of orange peels. It has been reported to possess pharmacological activities including analgesic, anti-inflammatory, antioxidant, anti-hypertensive, antiviral, and anti-carcinogenic effects. Hesperidin plays a role in the modulation of inflammatory markers and gene expression [7,8].

In this study, we investigated the effects of hesperidin on treatments administered in an experimentally-induced AOM rat model and evaluated the results both immunologically and histologically.

2. Material and methods

2.1. Animals and experimentally-induced AOM

In total, we used 28 healthy adult female albino Wistar rats (age: 3–4 months) obtained from the Experimental Animal Institute in Malatya-Turkey. Feed and drinking water was given ad libitum. Experiments were performed on the basis of the animal ethics guidelines outlined by the Institutional Animals Ethics Committee. The randomization was performed by all rats were 270–300 g body weight. For the groups, rat were selected randomly in all rats. Treatment groups were as follows: groups 2, 3, 4, and 5 had *S. aureus* strain ATCC 25923 in solution (0.5–1×10^8 CFU/ml) administered bilaterally using a dental needle (0.1 ml) via the transtympanic route. In experimental animals for AOM modeling *S. aereus* was used as Birdane et al. [9]. AOM developed within 48 h. Macroscopically, hyperemia of the tympanic membrane was observed on day 2, and growth of *S. aureus* was confirmed by culture. In earlier study, it was demonstrated that for the *S. aureus* proliferation and activation can be after 2 days of injection.

2.2. Treatment groups

Rats were randomly divided into five groups (n = 7 per group). Group 1 (control) rats served were given 0.1 ml saline via the intratympanic route, followed by 0.01% carboxymethilcellulose (CMC) using a gavage. In group 2 (AOM), AOM was induced by administering 0.1 ml solution of *S. aureus* via the intratympanic route, followed by 0.01% CMC using a gavage. In group 3 (AOM + antibiotic), AOM-induced rats were treated with 50 mg/kg antibiotic using a gavage. In group 4 (AOM + hesperidin), AOM-induced rats were treated with 100 mg/kg hesperidin using a gavage. In group 5 (AOM + hesperidin + antibiotic) AOM-induced rats were treated with 50 mg/kg antibiotic and 100 mg/kg hesperidin using a gavage. Hesperidin was dissolution in %0,01 CMC. It was demonstrated that CMC was no toxic and have side effect in rats. We used the amoxicillin for the treatment twice a day for 14 days. We used the hesperidin in treatment group for 14 days. Animals were sacrificed on day 14 under general anesthesia. Rats were weighted and then anesthetized with ketamine hydrochloride (75 mg/kg) and xylazine (8 mg/kg) was administered i.p. For immunological analysis, blood samples were collected from the left ventricle using an injector. Sera were obtained after whole blood centrifugation (3000 × *g*, 20 min, at 4 °C) and were stored at −45 °C until further analysis.

2.3. Cytokine analysis

Cytokine production was determined by enzyme-linked immunosorbent assays (ELISAs) using commercial kits according to the manufacturers' instructions. Interleukin-1beta (IL-1β) (cat no: EK0393) and IL-4 (cat no: EK0406), levels were measured using anti-rat ELISA kits from BosterBio (Pleasanton, CA, USA). IL-6 (cat no: KHC0061) and tumor necrosis factor-alpha (TNF-α) (cat no: KRC3011) were obtained from Invitrogen (Carlsbad, CA, USA). Microtiter plates were read at 450 nm using the CA-2000 ELISA microplate reader (CIOM Medical Co., Ltd., Changchun, China). Cytokine levels were calculated from standard curves of recombinant cytokines using a linear regression method.

2.4. Histopathological analysis

After rats were sacrificed, the temporal bone was dissected from the skull. The bullae were opened and tissues were placed in 10% buffered formalin fixative. For decalcification, tissues were incubated in 5% formic acid and routine tissue processing was performed. Each paraffin-embedded specimen was sectioned into 4 μm section and stained with hematoxylin and eosin (H&E). Slides were examined using a light microscope (Olympus BX-51; Olympus, Tokyo, Japan). Images were captured using a digital DP70 connected to a BX-51 microscope (Olympus). During the histological examination, thickening of the tympanic membrane (ThicTM), damage to the epithelium (DamEpith), inflammation (Inf), and sclerosis (Sc) were evaluated by light microscopy, semiquantitatively. [9]. The severity of changes were assessed using scores of none (−): normal epithelial and connective tissue; mild (+): mild infiltration of individual inflammatory cells or their clusters, mild degeneration of epithelial cells, mild connective tissue fibroblastic cell proliferation; moderate (++): moderate infiltration of inflammatory cells, focal epithelial loss and moderate connective tissue fibroblastic cell proliferation; and severe (+++): dense infiltration of inflammatory cells and loss of epithelial integrity and marked connective tissue fibroblastic cell proliferation. Statistical analysis was performed with numerical expressions of none (−)/0, Mild (+)/+1, moderate (++)/+2, severe (+++)/+3. Thic TM thickness was quantitatively assessed. The width of the tympanic membrane at the external edge facing the external auditory canal space and the width of the tympanic cavity facing the tympanic cavity were measured at 10 different points. The statistical results of the values are shown in Table 2.

2.5. Statistical analysis

Results were expressed as means ± standard deviation (SD). TNF-α, IL-4, IL-6, and IL-1β levels were compared among treatment groups using a one-way analysis of variance (ANOVA) and post-hoc Duncan test. Histological results were compared using a Kruskal–Wallis variance analysis. When differences among the groups were detected, group means were compared using a Mann–Whitney U test. We used the Mann–Whitney U test for histological parameters due to tympanic membrane thickness was suitable for this test. All analyses were carried out using SPSS for Windows software (ver. 18.0; SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant when the P-value was <0.01.

3. Results

Table 1 shows the serum levels of TNF-α, IL-4, IL-6 and IL-1β for all treatment groups. In the AOM group, serum levels of TNF-α, IL-4, IL-6 and IL-1β increased significantly compared to the other groups ($P < 0.01$). However, serum levels of TNF-α, IL-4, IL-6 and IL-1β were significantly decreased in the hesperidin, antibiotic, and hesperidin + antibiotic-treated groups compared to the AOM group. No significant differences in cytokine levels between the hesperidin, antiotic-treated and hesperidin + antibiotic groups were observed. In fact, these groups had cytokine levels similar to the control group.

3.1. Histopathological results

During the histopathological examination, changes in the tympanic membrane with regard to ThicTM, DamEpith, Inf and Sc are presented in Table 2 and Figs. 1–5. Existing changes are scored using semiquantitative evaluation. Their arithmetic mean is obtained as AOM and control groups are taken as the basis. In the control group, DamEpith, Inf, and Sc was not observed. Obvious increase in the ThicTM in AOM group is observed due to inflammation and edema effect. Dense inflammation is caused by infiltration of "neutrophils and lymphohistiocytic cells" (mean SD 2,83 ± 0,40). Due to inflammation, degenerative changes and cellularity increase are observed in the membrane wall's connective tissue. These changes occur secondary to the inflammation in the otitis media.

H&E stained sections showed that the values of ThicTM, DamEpith, Inf, and Sc were significantly increased in the AOM group compared to the control group (Table 2 and Figs. 1–5; $P < 0.001$).

For these parameters, the values in the AOM + antibiotic group were markedly lower than those observed in the AOM group (Table 2 and Figs. 2 and 3; $P < 0.001$).

Compared to AOM, in the AOM + hesperidin group, histopathological values were significantly decreased (Table 2, Figs. 2 and 4; $P < 0.001$).

As observed in Fig. 2 ThicTM in the AOM group increased with the migration of inflammatory cells and edema in the subepithelial layer of the bulla mucosa. In addition, the AOM group had the highest levels of DamEpith and Sc compared to all other groups. Between the AOM + antibiotic and AOM + hesperidin groups, there was a significant difference in ThicTM, Inf, and Sc values. However, no difference in DamEpith values was observed (Figs. 3 and 4). Finally, According to these parameters, the values in AOM + antibiotic + hesperidin group were markedly lower than those of the other groups (Table 2, Figs. 2–5). There was a significant difference in the AOM + antibiotic + hesperidin groups compared to AOM group (P < 0.001).

4. Discussion

AOM can occur on a scale of severity ranging from mild to severe, and is associated with reduced quality of life. Owing to its anatomical characteristics of being surrounded by bone tissue, the MEC is a site of poor antibiotic penetration. Antibiotics commonly used in the management of AOM include amoxicillin, amoxicillin/clavulanate, erythromycin base/sulfisoxazole, trimethoprim-sulfamethoxazole, cefixime, and cefuroxime axetil. Treatment with amoxicillin is prescribed for all children <2 years of age who are experiencing their first episode of AOM (1,3,7).

S. pneumoniae and *H. influenzae* are the main causes of AOM. The number of pediatric patients who are resistant to antibiotic therapy is increasing commensurate with an increase in the number of antibiotic-resistant bacteria [5]. Antibiotic resistance has guided medical researchers to investigate new options for the treatment of infectious diseases. Herbal remedies have been used for a long time and in fact continue to be used more often than prescribed medications [8–10]. For example, flavonoids are one of the most important constituents of fruit, especially in citrus species. Hesperidin is a flavanone glycoside, belonging to the flavonoid family, which has potent anti-inflammatory, anti-oxidant, and anti-cancer activities. More recent studies have indicated that hesperidin also has antimicrobial activity [11–13].

Table 1
Levels of various cytokines in rats from different treatment groups (n = 7, mean ± SD).

	IL-1 beta	IL-4	IL-6	TNF-a
Control	10,0 ± 6,51[a]	22,0 ± 5,67[a]	27,0 ± 2,06[a]	5,59 ± 2,69[a]
AOM	38,7 ± 8,06[b]	41,4 ± 8,50[b]	38,7 ± 2,56[b]	11,9 ± 2,67[b]
AOM + antibiotic	14,7 ± 1,33[ac]	25,2 ± 6,63[a]	27,6 ± 3,08[a]	3,45 ± 2,26[a]
AOM + hesperidin	19,7 ± 5,20[c]	23,2 ± 3,85[a]	26,2 ± 5,59[a]	4,40 ± 1,80[a]
AOM + hesperidin + antibiotic	17,5 ± 2,43[c]	22,9 ± 3.87[a]	26,8 ± 4,34[a]	3,98 ± 1,98[a]

SD, standard deviation; AOM, acute otitis media; IL, interleukin, TNF, tumor necrosis factor.
[a,b,c] Means bearing different superscripts within same column were significantly different ($P < 0.01$).

Table 2
Histopathological comparison of all treatment groups with regard to tympanic membrane thickness, epithelial damage, inflammation, and sclerosis.

	ThicTM	DamEpith	İnf	Sc
Control	16,33 ± 2,57	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00[a]
AOM	103,96 ± 3,23	2,66 ± 0,51	2,83 ± 0,40	2,50 ± 0,54
AOM + antibiotic	28,54 ± 5,26	0,66 ± 0,51[a]	0,66 ± 0,51	0,50 ± 0,54[a]
AOM + hesperidin	49,28 ± 6,23	0,66 ± 0,51[a]	1,33 ± 0,51	1,50 ± 1,22
AOM + hesperidin + antibiotic	25,13 ± 4,12	0,52 ± 0,50[a]	0,42 ± 0,13	0,30 ± 0,21[a]

[a] Means bearing different superscripts within same column were significantly different ($P < 0,01$). SD, standard deviation; AOM, acute otitis media; ThicTM, tympanic membrane thickness; DamEpith, epithelial damage; Inf, inflammation; Sc, sclerosis.

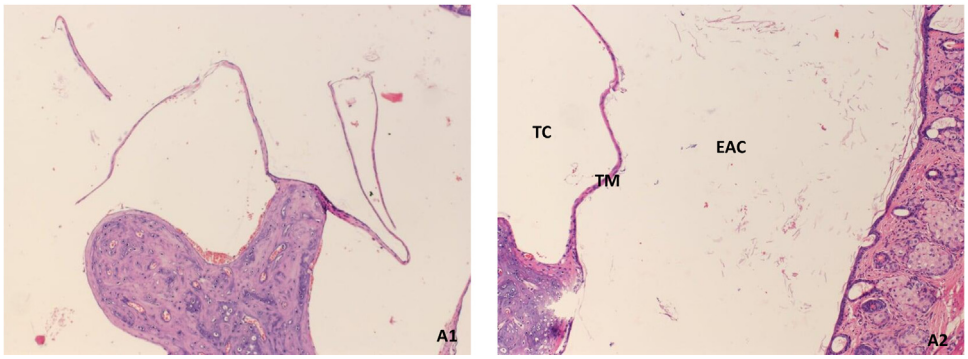

Fig. 1. Control group, A1: tympanic membrane structure and malleus attached to membrane in the control group A2: tympanic membrane structure in the control group, (A1; HE ×100, A2; HE ×200) EAC: external auditory canal, TC: tympanic cavity, TM: tympanic membrane, HE: hematoxylin and eosin. There were no characteristics in terms of the parameters evaluated in the control group.

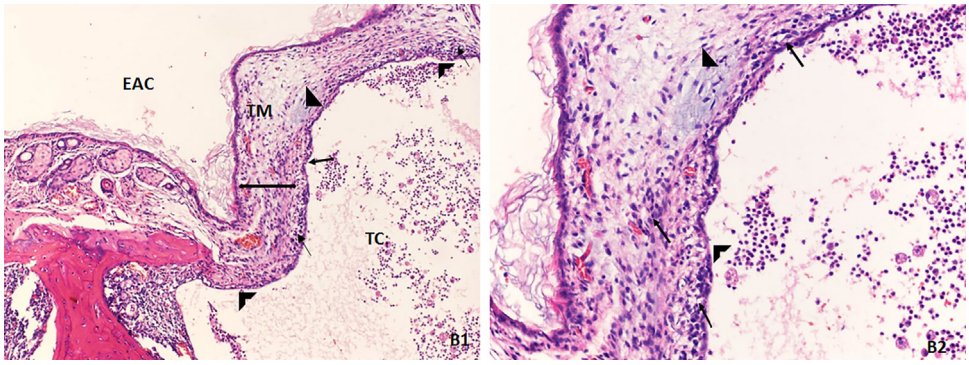

Fig. 2. Acute otitis media (AOM) group. Thickening of the tympanic membrane (↔), epithelial membrane damage (⌈), cellular inflammation (↑) of the connective tissue and sclerosis ( ), (B1 — HE ×100, B2 — HE ×400) EAC: external auditory canal, TC: tympanic cavity, TM: tympanic membran, HE: hematoxylin and eosin. ThicTM (score +3), DamEpith (score +3), Inf, and Sc (score +3).

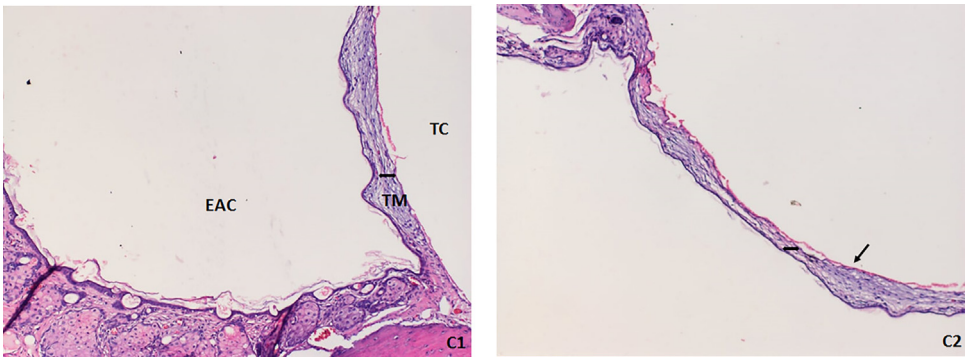

Fig. 3. Acute otitis media + antibiotic (AOM + antibiotic) group. The marked decrease in tympanic membrane thickening (↔), inflammation (↑) and sclerosis (C1 — HE ×100, C2 — HE ×200) EAC: external auditory canal, TC: tympanic cavity, TM: tympanic membran, HE: hematoxylin and eosin. ThicTM (score +1), DamEpith (score +1), Inf, and Sc (score +1).

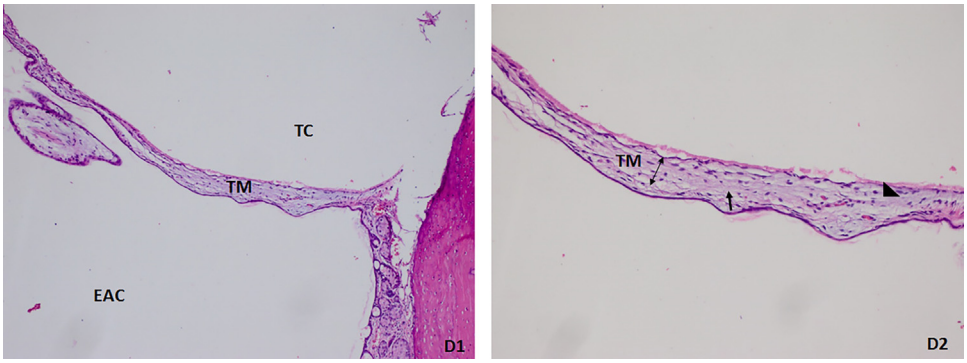

Fig. 4. Acute otitis media + hesperidin (AOM + hesperidin) group. The marked decrease in tympanic membrane thickening (↔), inflammation (↑) and sclerosis ( ), (D1 — HE ×100, D2 — HE ×200). EAC: external auditory canal, TC: tympanic cavity, TM: tympanic membran, HE: hematoxylin and eosin. ThicTM (score +1), DamEpith (score +1), Inf, and Sc (score +1).

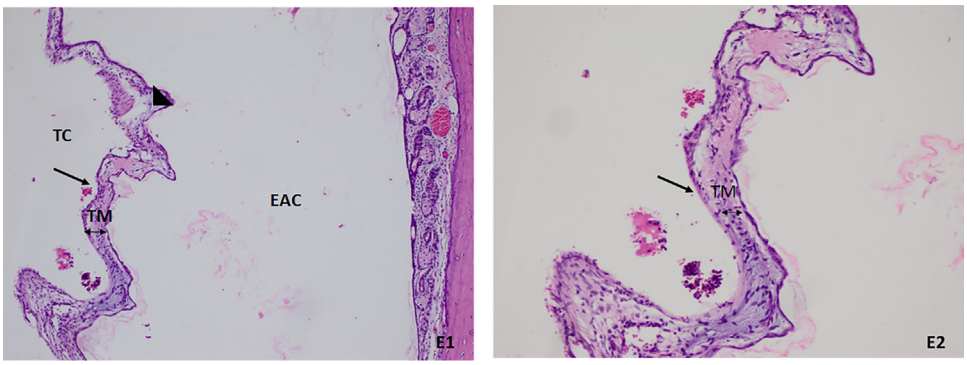

Fig. 5. Acute otitis media + antibiotic + hesperidin (AOM + antibiotic + hesperidin) group. The marked decrease in tympanic membrane thickening (↔), inflammation (↑) and sclerosis ( ) (E1 — HE ×100, E2 — HE ×200). EAC: external auditory canal, TC: tympanic cavity, TM: tympanic membran, HE: hematoxylin and eosin. ThicTM,DamEpith, Inf and Sc score none/0.

In this study, we investigated the use of a conventional antibiotic (amoxicillin) and a herbal medication (hesperidin), used in the management of anti-inflammatory and antimicrobial activities, to test their efficacy in the treatment of AOM. The effects of treatment were evaluated using four histopathologic examination parameters including ThicTM, DamEpith, Inf, and Sc [9]. In the AOM group, ThicTM increased with the migration of inflammatory cells and edema into the subepithelial layer of the bulla mucosa. DamEpith and Sc values were highest in the AOM group. Compared to the AOM group, all four values were significantly decreased in the AOM + hesperidin + antibiotic group. According to these parameters, the values in AOM + antibiotic + hesperidin group were markedly lower than those of the other groups. This effect may be due to synergistic effects occurring by using a combination of the antibiotic and hesperidin.

The role of cytokines in AOM has been well-studied using animal models. TNF-α, IL-10, IL-4, IL-6, and IL-1β are known as important local mediators associated with acute inflammation. The cytokine expression profile during AOM episodes is useful in evaluating disease pathogenesis [14–16]. In this study, we measured the serum levels of all four of these cytokines in the different treatment groups. Cytokine levels were significantly decreased in the hesperidin- and antibiotic-treated groups compared to the AOM group.

Several studies have revealed that hesperidin regulates the production of various inflammatory cytokines. In addition, hesperidin regulates inducible nitric oxide synthase, cyclooxygenase-2, by inhibiting its overexpression. These regulatory activities shed light on the potent anti-inflammatory effects of this herbal agent [8,17,18]. The role of hesperidin in hypolipidemia, in addition to its anti-hypertensive, anti-viral, anti-cancer and analgesic actions, has also been reported. Hesperidin is safe and has been approved by the Food and Drug Administration. Furthermore, at all doses tested, toxicity has not been observed [19,20].

In this prospective animal study, the effect of hesperidin on the treatment of acute otitis media was investigated in an acute otitis media-induced rat model. Histopathological and immunological analyses were performed and the results analyzed. To the best of our knowledge, we did not encounter a study that histologically and immunologically investigates the impact of hesperidin on the otitis media.

5. Conclusions

In this study, treatment of AOM with hesperidin for 14 days reduced AOM symptoms with similar efficacy to antibiotics. The favorable effects observed using hesperidin may be related to its ability to reduce oxidative stress, and to its anti-inflammatory activities. From our results, we propose that

hesperidin, in combination with antibiotics, may provide a successful alternative treatment for AOM compared with antibiotics used alone. For these reasons, we suggest investigating the potential use of hesperidin for the treatment of AOM. Future studies should focus on the use of hesperidin in the treatment of AOM using randomized controlled studies in humans.

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