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Canine atopic dermatitis: Role of luteolin as new natural treatment

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Abstract

Background: Luteolin has been demonstrated to possess numerous biological effects. However, the effect of luteolin on LPS (Lipopolysaccharides) stimulation in CPEK cells has not been investigated.

Hypothesis/Objectives: An in vitro model of atopic canine dermatitis was used to identify the antioxidant effect of luteolin as a new treatment that is capable of improving the conditions of veterinary patients.

Methods: CPEK cells were treated with or without luteolin in the presence or absence of LPS. A cell viability assay was performed to test luteolin toxicity and the protective effect of luteolin after LPS stimulation. Additionally, enzyme-linked immunosorbent assay (ELISA) kits were used to detect the levels of IL-33, IL-1β, IL-6, and IL-8.

Results: Luteolin was capable to significantly decrease levels expression of IL-33, IL 1β, IL-6, and IL-8.

Conclusions and clinical importance: Luteolin could be a new pharmacological treatment for canine atopic dermatitis.

KEYWORDS

Canine atopic dermatitis, in vitro model, inflammation, Luteolin

1 | INTRODUCTION

Canine atopic dermatitis (cAD) is considered a chronic inflammatory and pruritic disease that affects the skin with a diversity of clinical signs. This disease has been observed as a spontaneous atopic animal condition because domestic dogs share the environment with humans, and cAD shares many clinicopathological features with human AD (hAD). Sometimes, a genetic predisposition is present; the disease is also recurrent and affects 10% of the canine population. There are breeds that are more prone than others to developing this disease, and so the percentage of sick subjects is increased. In the veterinary field, cAD is a disease that affects both the quality of life of affected dogs and that of their owners (Halliwell, 2006; Majewska et al., 2016). cAD is characterized by a multifaceted pathogenesis that is still not completely clarified. cAD principally affects young dogs and frequently perseveres during their entire life; however, there is no exact information on the natural history of cAD. Both environmental and genetic factors are active in the development of the clinical disease. The first step of development involves sensitization to environmental allergens localized in the space, particularly in the house, as dust and mites are able to penetrate the skin and lead to the recruitment and stimulation of resident inflammatory cells and degranulation of mast cells via binding to IgE. Moreover, inflammatory mediators,

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such as cytokines, particularly type 2 cytokines and chemokines, are secreted, regulating the progression of the pathology. Among cytokines, interleukin (IL)-33 has been implicated in the pathogenesis of atopic diseases, particularly in AD. This cytokine is mainly expressed as a nuclear protein by endothelial cells and keratinocytes in the skin. IL-33 belongs to the IL-1 cytokine family (Lloyd & Hessel, 2010), in vitro, it induces the expression of IL-13 and IL-5 and Th2 cytokines in vivo, IL-33 also induces splenomegaly and increases the number of eosinophils in blood and the expression of serum immunoglobulins (Schmitz et al., 2005). Moreover, IL-33 is liberated by physical stress or tissue damage and functions as an endogenous danger signal. Furthermore, T cells and IL-33 are also able to stimulate both the activation and maturation of human mast cells (Allakhverdi, Smith, Comeau, & Delespesse, 2007). A human study showed that IL-33 mRNA levels are increased approximately 10-fold in the skin of atopic dermatitis (AD) patients compared to those of healthy skin (Pushparaj et al., 2009). In mice, subcutaneous injection of IL-33 caused cutaneous fibrosis that was related to eosinophils and IL-13 but not IL-4 (Brandt & Sivaprasad, 2011; Rankin et al., 2010). However, the role of IL-33 in atopic dermatitis is still under consideration. In controlled clinical trials, it was demonstrated that both oral and topical glucocorticoids and oral microemulsified cyclosporine are classified as *drugs of choice* in the management of cAD. (Olivry & Bizikova, 2013). As pharmacological substances, these drugs have adverse side effects depending on both the dose and regimen employed. It is well known that the combined treatment of different drugs is sometimes more effective than using only one therapy, since it reduces the appearance of side effects (Cain, 2019). On the basis of what is known in the literature and the state of health of the animals affected by this pathology, it is important to study new and alternative treatments to be used alone or in a combined therapy regimen. Based on this, we decided to investigate the role of an antioxidant substance that belongs to the flavonoid family. Luteolin (3′,4′,5,7-tetrahydroxyflavone) is one of the most powerful and effective polyphenols in vegetables, fruits and medicinal herbs (Nabavi et al., 2015). Luteolin has several biological properties, such as anticancer, antioxidant, neuroprotective and anti-inflammatory effects, which have been shown in both in vitro and in vivo models (Chen et al., 2008; Cheng et al., 2010; Dirscherl et al., 2010, 2012; Kang, Lee, Choi, Kim, & Han, 2004; Lin, Shi, Wang, & Shen, 2008; Pandurangan & Esa, 2014; Zhang, Gan, Shelar, Ng, & Chew, 2013). Several studies have demonstrated that luteolin inhibits nuclear factor kappa B (NF-κB) signalling, cytokine expression and TLR4 signalling at micromolar concentrations in immune cells, including mast cells (Kim & Jobin, 2005; Lee et al., 2009; Weng, Patel, Panagiotidou, & Theoharides, 2015). Furthermore, luteolin inhibits the Keap1-Nrf2-ARE pathway in PC12 cells (Lin, Wu, Liu, Su, & Yen, 2010). Although the action of various antioxidants in cAD has been studied, nothing is known about the effects of luteolin. Our study aimed to evaluate the action of luteolin in an in vitro model of atopic canine dermatitis to identify whether this antioxidant is a new treatment that is capable of improving the conditions of veterinary patients.

2 | MATERIALS AND METHODS

2.1 | Cell culture and treatment

The proliferative canine keratinocyte cell line (CPEK; CELLnTEC, Zen-Bio Inc) was cultured in 25 cm^2 flasks (Sigma-Aldrich) in CnT-09 (CELLnTEC Advanced Cell Systems) with 10% foetal bovine serum (FBS; Sigma-Aldrich) and 1% antimycotic antibiotics (Thermo Fisher Scientific Inc) at 37°C and 5% CO_2 until approximately 80%–85% confluence. The cultured cells were trypsinized by treatment with 2 ml Trypsin-EDTA and then incubated for 7–10 min at 37°C. After adding media and washing with sterile phosphate-buffered saline (PBS), the cells were resuspended in CnT-09 media, counted with trypan blue, and plated on sterile 6- or 12-well cell culture plates.

As previously described by Mullin et al., to stimulate CPEK cells, 1 ml of 20 ug/ml LPS (Sigma-Aldrich) or CnT-09 alone was added to the wells, and the plates were incubated for an additional 24 hr (LPS) at 37°C before harvesting (optimization data not shown)(Mullin, Carter, Williams, McEwan, & Nuttall, 2013). Fifth- to 10th-passage CPEK cells were used in these experiments, and all conditions were performed in triplicate.

CPEK cell cultures were divided into the following groups:

1. CTR: cells cultured with normal culture medium;
2. LPS: cells stimulated with LPS; and.
3. LPS + Dex: cells stimulated with LPS and treated with Dexamethasone (Dex).
4. LPS + Lut: cells stimulated with LPS and treated with different concentrations of Lut.

Dexamethasone (1 μM) was employed as positive control (Rodriguez-Luna et al., 2017) (Werner, Braun, & Kietzmann, 2008).

Luteolin (purity > 98%) was purchased from Sigma Chemical (St. Louis., MO, USA, Cat#: 491–70–3). The luteolin concentrations were chosen based on a previous study conducted by Zhang, Li, Xu, Xiang, and Ma (2018).

2.2 | Vital staining

To assess the viability of cell cultures, after LPS treatment alone or with different concentrations of luteolin for 6 hr, the cells were incubated at 37°C with 0.2 mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) for 1 hr. The optical density at 550 nm (OD550) was measured using a microplate reader and used to calculate the cell viability (Paterniti et al., 2017).

2.3 | Measurement of cytokine production

Medium samples were evaluated for IL-33, IL-1β, IL-6 and IL-8 by ELISA according to the manufacturer's instructions. The absorbance was read at 450 nm, and the background wavelength correction was set at 540 nm or 570 nm (Paterniti et al., 2017).

2.4 | Materials

Unless otherwise specified, all compounds were obtained from Sigma-Aldrich. All other chemicals were of the highest commercial grade available. All stock solutions were prepared in nonpyrogenic saline (0.9% NaCl, Baxter, Milan, Italy).

2.5 | Statistical analysis

The data were analysed by one-way ANOVA followed by a Bonferroni post hoc test for multiple comparisons. A p-value of less than .05 was considered significant. Data are representative of at least three independent experiments. #$p < .05$ versus LPS; **$p < .01$ versus CTR; ##$p < .01$ versus LPS; ***$p < .001$ versus. CTR; ###$p < .001$ versus LPS.

3 | RESULTS

3.1 | Effect of Luteolin on CPEK cell viability

To understand whether luteolin exerts a toxic effect on CPEK cell viability, we performed an MTT assay. As shown in Figure 1, CPEK cells were incubated with different concentrations of luteolin (from 0 μM to 128 μM) for 24 hr. We found that at concentrations of 16 μM, 32 μM, 64 μM or 128 μM, luteolin significantly decreased cell viability. On the other hand, CPEK cell viability was not affected by luteolin at concentrations less than 8 μM. Numerical data: 1 μM (98.80% ± 0.99), 2 μM (98.80% ± 0.88), 4 μM (95.30% ± 1.46), 8 μM (94.70% ± 1.32), 16 μM (85.50% ± 4.61), 32 μM (72.50% ± 1.97), 64 μM (55.30% ± 2.05) and 128 μM(42.10% ± 2.79).

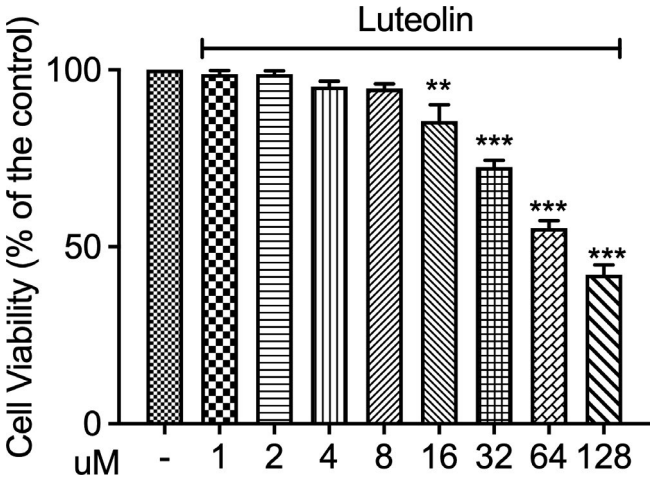

FIGURE 1 Effect of Luteolin on CPEK viability. Cell viability was evaluated using MTT tetrazolium dye. Concentration higher 16 μM, significantly decreased cell viability. **$p < .01$ versus. control; ***$p < .001$ versus. control

3.2 | Protective effect of luteolin on CPEK cells after LPS stimulation

Because CPEK cell viability was compromised by luteolin at concentrations greater than 8 μM, we continued our experiments with lower concentrations (1 μM, 2 μM, 4 μM and 8 μM). To investigate the cytoprotective effect of luteolin, we performed an MTT assay after LPS stimulation. As shown in Figure 2 we found that luteolin significantly decreased LPS-induced cell death. Numerical data: Control (100%), LPS (54%±5.50), Dex (95,26 ± 1,69), 1 μM (91%±2.67), 2 μM (93%±2.83), 4 μM (77.20%±6.12) and 8 μM (76%±3.90).

3.3 | EFFECT OF Luteolin On IL-33, IL-1β, IL-6 And IL-8 Release

Since IL-33 plays important roles in atopic disease, we investigated the effect of luteolin on the release of IL-33 by CPEK cells. As shown in Figure 3a, luteolin significantly inhibited the release of IL-33 at a concentration of 1 μM. Because the IL-33 and IL-1β families are related by origin, receptor and signalling pathways, we also investigated the effect of luteolin on IL-1β secretion. We found that the release of IL-1β was significantly inhibited at a concentration of 1 μM (Figure 3b).

We also evaluated the effect of luteolin on IL-6 and IL-8 and found, similarly, that a very low concentration of luteolin decreases the release of IL-6 and IL-8. IL-33 numerical data: Control (17.36 pg/ml ± 1.09), LPS (41.91 pg/ml ± 2.90), Dex (22.45 pg/ml ± 3.43), 1 μM (26.73 pg/ml ± 2.51), 2 μM (26.91 pg/ml ± 2.66),

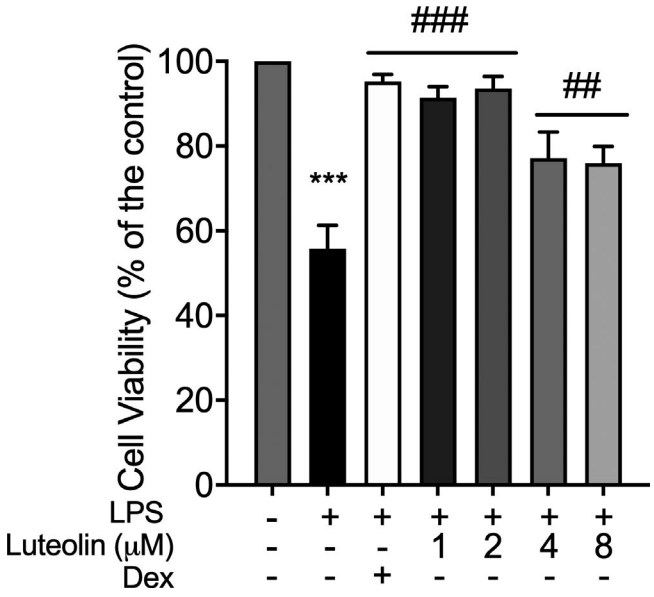

FIGURE 2 Protective effect of luteolin on CPEK cells after LPS intoxication. Protective effect of luteolin was determined after 24 hr of LPS using MTT tetrazolium dye. Luteolin was able to significantly decrease cells death LPS-induced ate the concentration of 1 μM, 2 μM, 4 μM, 8 μM. ##$p < .01$ versus, LPS; ***$p < .001$ versus control; ###$p < .001$ versus LPS

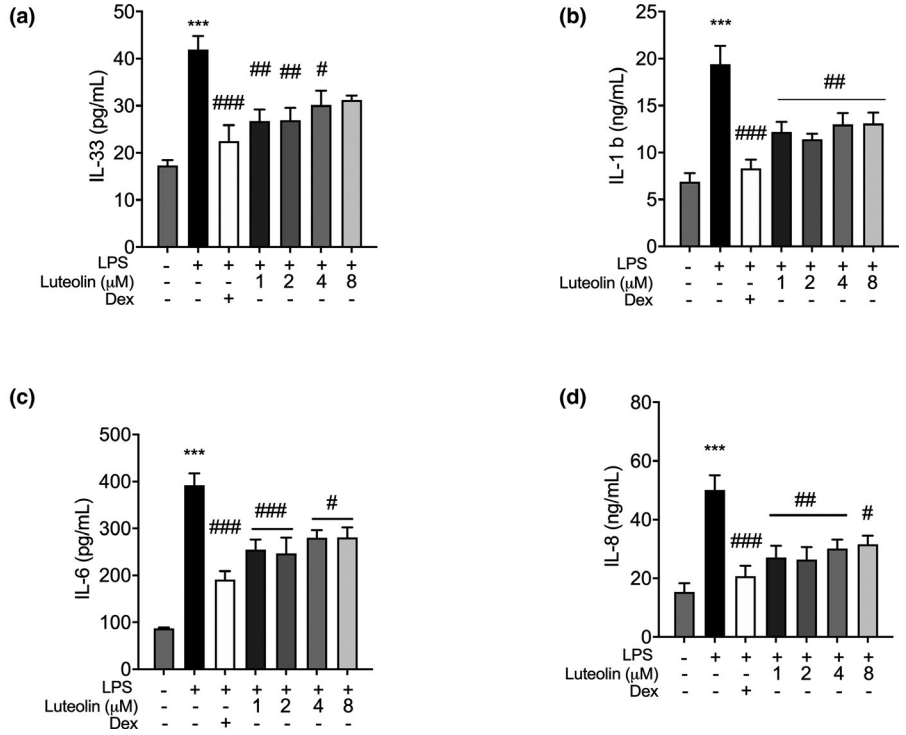

FIGURE 3 Effect of Luteolin on IL-33, IL 1β, IL-6 and IL-8 release. ELISA quantification of IL-33 (a), IL 1β (b), IL-6 (c) and IL-8 (d) after LPS intoxication and luteolin treatment. Luteolin was capable to significantly decrease IL-33 (a), IL 1β (b), IL-6 (c) and IL-8 (d) production. #$p < .05$ versus LPS; **$p < .01$ versus control; ##$p < .01$ versus LPS; ***$p < .001$ versus control; ###$p < .001$ versus LPS

4μM (30.16 pg/ml ± 3.05) and 8 μM (31.20 pg/ml ± 0.95); IL 1β numerical data: control (6.90 ng/ml ± 0.90), LPS (19.40 ng/ml ± 1.96), Dex (8.31 ng/ml ± 0.92), 1 μM (12.20 ng/ml ± 1.05), 2 μM (11.40 ng/ml ± 0.60), 4μM (13.00 ng/ml ± 1.20) and 8 μM (13.10 ng/ml ± 1.16); IL-6 numerical data: control (87.41 pg/ml ± 1.36), LPS (392.25 pg/ml ± 25.06), Dex (191 pg/ml ± 18.00), 1 μM (254.60 pg/ml ± 22.03), 2 μM (247.20 pg/ml ± 33.57), 4μM (280.40 pg/ml ± 15.75) and 8 μM (281.0 pg/ml ± 21.28); IL-8 numerical data: control (15.40 ng/ml ± 2.93), LPS (50.10 ng/ml ± 5), Dex (20.77 ng/ml ± 3,5), 1 μM (27.10 ng/ml ± 3.96), 2 μM (26.40 ng/ml ± 4.22), 4μM (30.10 ng/ml ± 3.10) and 8 μM (31.60 ng/ml ± 2.96).

4 | DISCUSSION

cAD is a common chronic, worsening pruritic skin disease in dogs for which treatment changes in relation to time and geographical location. Because of the diversity of the phenomena involved in the development of cAD and the different clinical aspects, a supplementary rational and personalized therapeutic approach is necessary for each patient. Chronic skill inflammation, persistent skin infections and pruritic manifestations are the clinical aspects of cAD(Santoro, 2019). It was observed, in particular as clinical usual signs a general pruritus accompanied by erythema, papules, pustules, crusts and excoriations (Hensel, Santoro, Favrot, Hill, & Griffin, 2015). Four are the principal factors regarding cAD treatment; in particular: time, inflammation, pruritus and finally infections. Both chronicity and severity of lesions establish the choice of short-term or long-term medications, considering also efficacy, side effects and related costs (Santoro, 2019).The therapeutic approach should be modified for every atopic animal, respecting the needs of each dog and the dog's owners. Today, the cornerstone prescribed drugs are glucocorticoids that are commonly linked to an important decrease in inflammation and pruritus.These are flexible compounds that can be employed both systemically and topically; in particular, literature data reported that topical treatment with GC have been employed during the past decades for the reduced presence of systemic side effects if compared with oral GCs administration; nevertheless, in particular cutaneous atrophy and calcinosis cutis represent the probable side effects for several topical GC treatment (Saridomichelakis & Olivry, 2016).Most of the clinical studies referenced here also showed that glucocorticoids have significant side effects, such as hyper adreno corticism and Cushing's disease (Olivry & Sousa, 2001). For this reason, it is currently necessary to find new molecules without any or with minor side effects. Recently, several studies have described a panel of pro-inflammatory cytokines that play an essential role in both the induction and maintenance of chronic skin inflammation. In the presence of specific subgroups of Th lymphocytes, the response is polarized, and keratinocytes become targets downstream of these cytokines. The cytokine environment plays a relevant role in both skin morphology and innate immunity, and keratinocytes are players in innate immunity (Bernard et al., 2012). IL-33 is classified as member of the IL-1 family of cytokines that has been involved in the pathogenesis of several atopic diseases, such as AD and allergic asthma (Cevikbas & Steinhoff, 2012).

Several types of cells are able to express IL33 and among these in particular epithelial, mast and dendritic cells (Liew, Pitman, & McInnes, 2010). In skin, IL-33 is principally detected as a nuclear protein in keratinocytes and endothelial cells (Sundnes et al., 2015). Tissue damage or physical stress act as endogenous danger signals and release this cytokine (Kakkar, Hei, Dobner, & Lee, 2012). Previous research has shown that keratinocytes express IL-33 in the lesional skin of human AD (Du et al., 2016). Moreover it was demonstrated that serum IL-33 levels were considerably higher in patients with AD than in healthy controls and associated with gravity scores (Schmitz et al., 2005). IL-33 is predominantly expressed as a nuclear protein by both keratinocytes and endothelial cells in skin (Sundnes et al., 2015) and is released by tissue damage or physical stress, which functions as an endogenous danger signal. Research on alternative and new therapies for management of cAD has increased in the past year. Moreover, investigators have invested in examining more natural compounds with fewer side effects that are able to decrease or totally remove the needs for medications that are usually used for AD. Natural products have been extensively used for the management of chronic skin diseases, such as psoriasis and AD (Shu, 1998). In particular, flavonoids are usual constituents of plants employed in traditional medicine to manage a varied range of diseases (Panche, Diwan, & Chandra, 2016). The class of flavonoids includes a large group of plant secondary metabolites depicted by a diphenylpropane structure (C6-C3-C6). They are extensively distributed throughout the plant kingdom and are frequently found in vegetables and fruits (Prasain & Barnes, 2007). Luteolin is a naturally occurring polyphenolic flavone that exists as glycosides in vegetables and fruits. Epidemiological findings proposed that a LUT-rich plant-derived diet may play a significant role in the reduction of many diseases through the pharmacological activity of luteolin, such as antimicrobial, anti-inflammatory, antioxidant, anti-allergic, anticancer and anti-platelet properties (Lopez-Lazaro, 2009; Zhang, Yang, & Wang, 2016). With this background in mind, we aimed to specifically block new cytokines as a different approach for the treatment of AD. As a first step, we evaluated luteolin toxicity in CPEK cells and found that luteolin was toxic at concentrations higher than 16 μM. After LPS stimulation, luteolin showed an important protective effect on cell viability. Luteolin decreased LPS-induced cytotoxicity at very low concentrations.IL-33 plays central roles in atopic diseases and organizes the activation of various ST2-expressing structural cells and haematopoietic cells. For this reason, we investigated the effect of luteolin on the release of IL-33 by CPEK cells. We found that luteolin significantly inhibited the release of IL-33 and IL-1β as well as IL-6 and IL-8. These studies open the way to identifying new therapeutic strategies focused on more specific objectives, such as keratinocyte-targeting cytokines, rather than on the systemic inhibition of T lymphocyte-mediated cytokine production. Atopic canine dermatitis is an insidious disease with many clinical aspects and has always been a source of considerable frustration both for the veterinary surgeon and for the owner of the animal because of the difficulties in diagnostic and therapeutic management, as well as the low probability of safe and definitive success. Today, the only weapon we can rely on to control atopic dermatitis is knowledge of the different clinical pictures and the most innovative therapeutic approach. It is important to emphasize that the treatment should take into account the duration and severity of the symptoms, the response to therapeutic protocols already adopted and the economic availability of the owner. Because cAD is a chronic disease that accompanies the subject throughout life, it is almost always impossible to avoid contact with allergens. In the past decade the investigation of unconventional therapies to treat canine AD has seen a remarkable intensification. Researchers have examined several natural compounds with fewer side effects able to reduce or totally eradicate the needs of medications usually used for AD. Fortunately, in recent years, the therapeutic tools that are able to control atopic symptoms have greatly improved and, therefore, allow the animal to better coexist with its allergic state. Antihistamines, corticosteroids, ALIA mechanism molecules, essential fatty acids, immunomodulators and antioxidants are the most commonly used substances in atopic subjects. These treatments and the control of allergenic exposure or concomitant infections, hyposensitization or specific topical treatments are essential weapons to reduce pruritic symptoms, check their periodic recrudescence and improve the quality of dog's life. In the future the hope is to identify even safer and more tolerated treatments for the management of canine dermatitis. The options of treatment should be frequently reviewed and changed considering the individual necessities of both veterinary patients and their owners. It is universally recognized that the best treatment for cAD associates different characteristics such as great efficacy and low cost and minor side effects.

CONFLICT OF INTEREST

The authors report no conflict of interest.

AUTHOR CONTRIBUTION

Enrico Gugliandolo: Conceptualization; Writing-original draft. **Ernesto Palma:** Data curation; Formal analysis. **Marika Cordaro:** Conceptualization; Writing-original draft. **Ramona D'amico:** Data curation. **Alessio Filippo Peritore:** Formal analysis. **Patrizia Licata:** Writing-review & editing. **Rosalia Crupi:** Supervision; Writing-review & editing.

PEER REVIEW

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