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Short Communication

β-sitosterol ameliorates inflammation and *Pseudomonas aeruginosa* lung infection in a mouse model

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ABSTRACT

We previously demonstrated that β-sitosterol (BSS) inhibits the expression of the chemokine IL-8 in CF bronchial epithelial cells exposed to *P. aeruginosa*. In the mouse model of lung chronic infection, herein shown, BSS significantly reduced leukocyte recruitment in the bronchoalveolar lavage fluid and decreased bacteria recovered in the airways. Treatment with BSS decreased the expression of key cytokines involved in immune response, mainly neutrophil chemotaxis, in the lung homogenate. This anti-inflammatory activity is accompanied by a beneficial protecting activity against infection and improvement of health status. Our data suggest that BSS has the potential to become a new drug to target the excessive neutrophil recruitment in lungs chronically infected by *P. aeruginosa* and encourage future investigations on mechanism of protection driven by BSS.

New Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) modulators of defective protein are having a positive impact on Cystic Fibrosis (CF) care [1]. F508del-CFTR pwCF treated with the potent modulator Trikafta (Kaftrio) feel significant benefits of lung function with reduction of inflammatory markers [2]. Whether mutant CFTR modulators are sufficient to completely halt and report to healthy baseline levels lung inflammation in pwCF adults and adolescents with advanced lung disease is presently under scrutiny and novel anti-inflammatory strategies continue to be proposed (for update see Editorial [3] and the related Research Topic collection of articles). Conversely, whether CF lung infection and inflammation augment or reduce the efficacy of mutant CFTR modulators is also controversial, considering the heterogeneity of the CF airway surface pathophysiology (for recent review see [4]). We previously demonstrated that β-sitosterol (BSS) extracted from *Nigella arvensis L.* seeds, broadly used as anti-inflammatory remedies in traditional medicine of Northern Africa, inhibits the expression of the pro-inflammatory neutrophil chemokine Interleukin (IL)−8 in CF bronchial epithelial cells exposed to *P.aeruginosa* [5]. BSS is one of the most abundant sterols deriving from plants, widely tested for efficacy and safety in clinical pharmacology with many potential applications as anti-microbial, anti-inflammatory, or immunomodulatory agent [6]. Very importantly, BSS is one of the bioactive compounds of traditional Chinese herbal medicine against respiratory diseases [7]. We hypothesized that BSS can be used to reduce the inflammatory response in CF. Preclinical evaluation can be performed in models with varying degrees of disease severity (acute and chronic). First, BSS was tested in the mouse model of lung acute infection showing that this treatment significantly improved the health status of mice (supplementary figure 1A). Total and differential cell counts in the bronchoalveolar lavage fluid (BALF) showed a trend for reduction (supplementary figure 1B), which was not statistically significant after a single treatment, indicating that repeated administrations may be required to demonstrate therapeutic efficacy. Significant reduced bacterial burden in the airways was also observed (supplementary figure 1C). Next BSS was tested in the mouse model of lung chronic infection that mimics the advanced stage of lung pathology in humans [8,9]. Chronic infection is usually established by including

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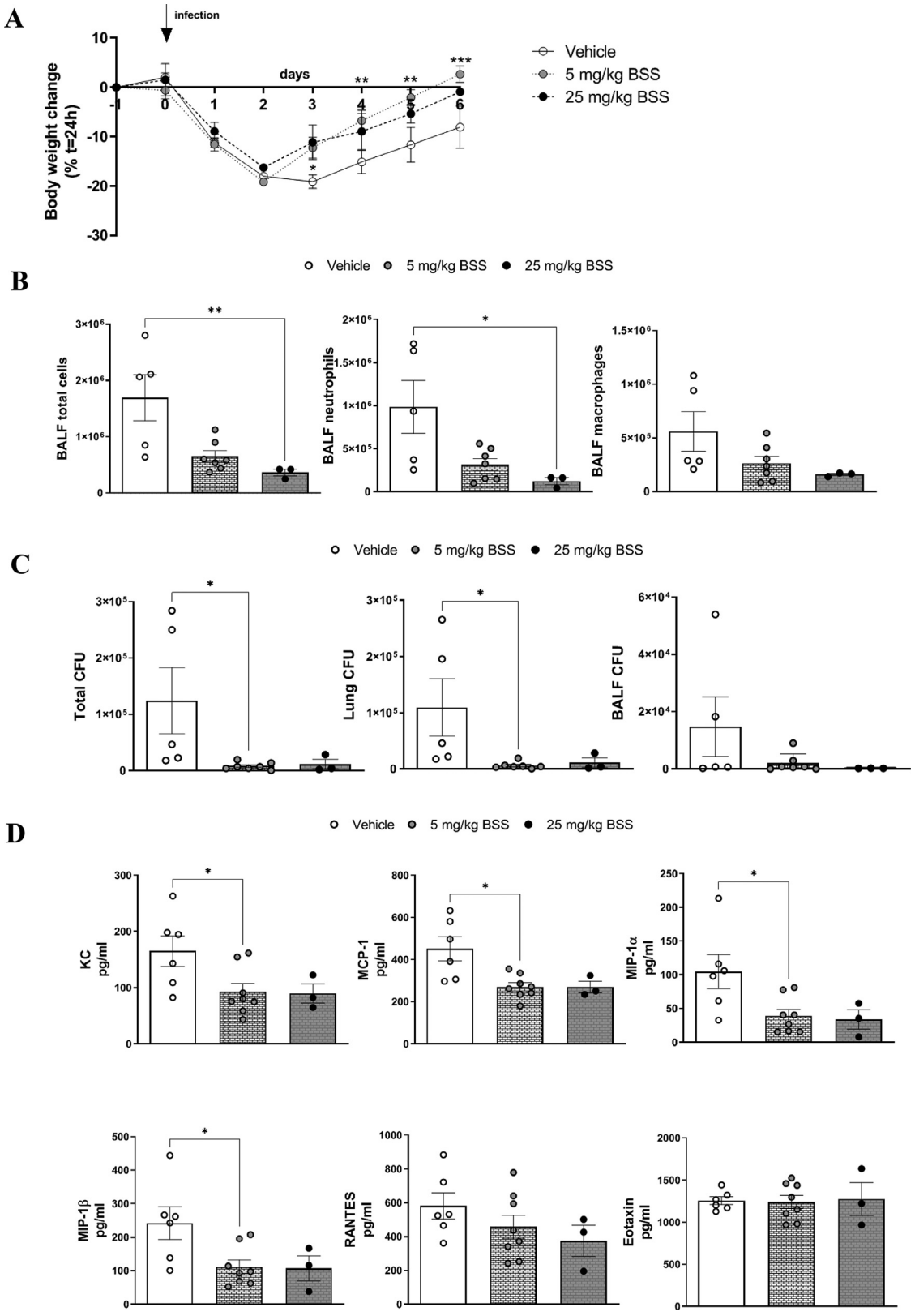

Fig. 1. Effect of BSS in a murine model of *P. aeruginosa* chronic infection. C57Bl/6NCr male mice were infected with *P. aeruginosa* MDR-RP73 embedded in agar beads by intratracheal inoculation (4–5 × 10^5 CFU). Treatment with 5 and 25 mg/Kg BSS or vehicle by gavage was started 24 h before and daily for 6 days after infection. A: percentage change from the initial body weight. Weight of mice were recorded before (t=−1) and after infection, daily for 6 days. Comparison between groups were made by two way ANOVA with Bonferroni post-test. Data reported are mean ± SEM of two different experiments. n = 5 (vehicle), 7 (5 mg/Kg) and 3 (25 mg/Kg). B: Inflammatory response in BALF. Total cells, neutrophils and alveolar macrophages recruited in BALF 6 days after infection were counted as detailed in Supplementary. n = 5 (vehicle), 7 (5 mg/Kg) and 3 (25 mg/Kg). C: Infection in BALF and lung. Total, lung and BALF CFUs are shown. n = 5 (vehicle), 7 (5 mg/Kg) and 3 (25 mg/Kg). D: Chemokine concentrations in supernatant of lung homogenate. E: cytokines. F: growth factors. Data are presented as the mean ± SEM pooled from two independent experiments. n = 6 (vehicle), 8 (5 mg/Kg) and 3 (25 mg/Kg). Comparisons between groups were made by non-parametric Kruskal Wallis test.

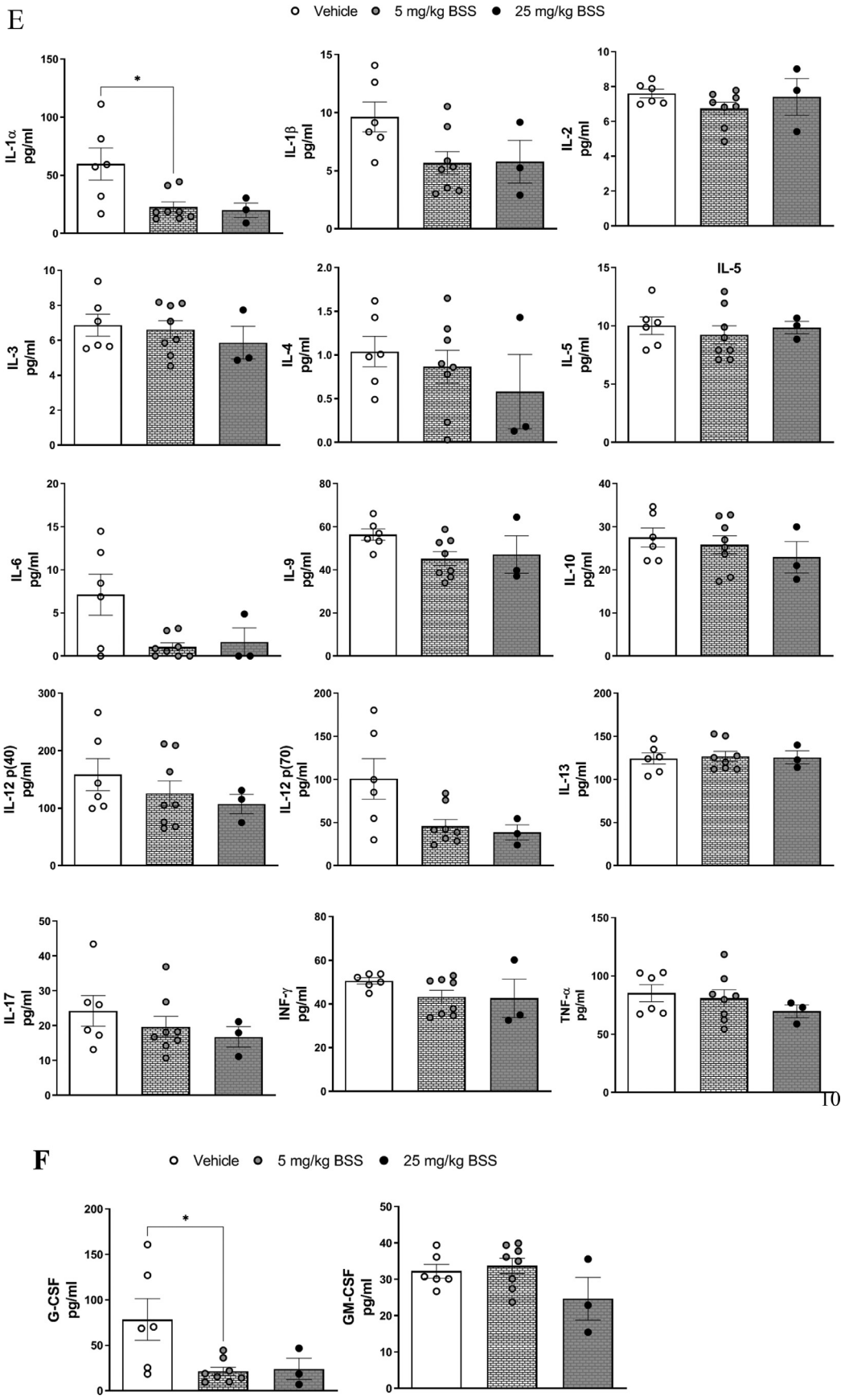

Fig. 1. Continued

CF-adapted bacterial variants in immobilizing agents, where they grow in microcolonies under micro-aerobic/anaerobic conditions, as in the mucus of pwCF [8,9]. *P. aeruginosa* persistence in this model has a greater effect on inflammation and damage profile rather than CFTR mutation itself and can be used in pre-clinical studies for CF. For the chronic infection, *P. aeruginosa* MDR-RP73 clinical strain embedded in agar beads was prepared as described [8] and detailed in online data supplement. Twenty-four hours before infection, C57Bl/6NCr male mice were treated with 5 mg/Kg or 25 mg/Kg BSS by oral gavage. Mice were monitored for body weight and treatment was extended daily for 6 days. BSS treated mice exhibited significant faster recovery of body weight than vehicle-treated mice, thus suggesting an improvement of health status (Fig. 1A). When mice were analysed six days after infection, a reduction of leukocyte recruitment in BALF of mice treated with BSS was observed (Fig. 1B). Both neutrophils and alveolar macrophages were decreased in BSS treated mice. To verify that reducing inflammatory cells in chronically infected mice does not impair host defense or exacerbate infection, bacterial load was evaluated in the airways of mice, including BALF and lung. A decrease of bacteria recovered in the airways of mice was found, thus indicating an ameliorating effect of BSS on infection (Fig. 1C). As we have previously shown that addition of BSS in broth culture of *P. aeruginosa* strain PAO1 does not result in bactericidal and bacteriostatic effects [5], the decrease of infection in BSS-treated murine lungs could be related to a more effective host immune response. Independently of the mechanism, our findings indicate that BSS improves the ability of mice to fight against chronic infection. Since the modulatory response of inflammatory cascade is crucial for clearance of microbial agents and equally important to ensure that infection does not cause excessive tissue injury, the effect of BSS on production of chemokines, cytokines and growth factors was evaluated in lung homogenate. BSS-treated mice showed a reduction of the typical chemokines activated by bacterial infection such as KC, MCP-1, MIP-1α and MIP-1β in the lung (Fig. 1D). IL-1α, the pro-inflammatory cytokine, playing a central role during infection was also decreased in BSS-treated mice (Fig. 1E). Moreover, the cytokines IL-6 and IL-12(p70) and the growth factor of granulocytes G-CSF were lowered (Fig. 1F). These data demonstrate that treatment with BSS reduces the expression of key cytokines involved in immune response, mainly neutrophil chemotaxis, in mice chronically infected with *P. aeruginosa.* The precise mechanism of anti-inflammatory action of BSS is presently not understood. What we previously found in a CF bronchial epithelial cell model exposed to *P.aeruginosa* is that BSS inhibits the activation of the calcium-dependent classical isoform alpha of Protein Kinase C (PKC) [5]. This is consistent with the interaction of *P.aeruginosa* with bronchial epithelial cells inducing the release of "danger signals", as ATP and UTP that, ligating PY2 purinergic receptor, activates the release of calcium from endoplasmic reticulum and PKC-dependent activation of transcription factors like NF-kB, finally promoting expression of several chemokines and pro-inflammatory cytokines. Whether this represents the main mechanism of anti-inflammatory action of BSS requires further investigation. Most importantly, this anti-inflammatory activity is accompanied by a beneficial protecting activity against infection. Anti-infective effect of BSS, not necessarily dependent on its unlikely bacteriostatic or bactericidal effect, has been reported in different models [6,7], including those from our group [5]. Although this effect has not been yet extensively investigated, it has been shown to influence virulence factors by inactivating bacterial toxins or inducing anti-bacterial responses of the host, as it protects mice from lethal infection by *Streptococcus pneumonie*, by interacting with pneumolysin [10], regulates invasion and survival of *Brucella abortus* via nitric oxide and cytokine production [11] or alleviates *Salmonella typhimurium* induced colitis by increasing expression of antimicrobial peptides [12]. In addition, BSS has been shown to modulate the adaptive immune response against infection in different experimental models, *e.g.,* activation of M1 macrophages through Th1 response [13] and activation of dendritic cells [14]. All these reports encourage future investigations on mechanism of protection driven by BSS on murine model of *P. aeruginosa* chronic lung infection presented here. Anti-inflammatory effect of phytosterols has been demonstrated in several animal models [6], and promising immune modulating properties have been found in clinical trials in patients with chronic infections such as pulmonary tuberculosis, by HIV or Human Papilloma Virus suggesting them as adjuvants to conventional pharmacological treatments or alternative drugs [15]. The doses used here, reflecting potential dosages in humans, are compatible with those used by Racette [16], showing no adverse effects up to a daily intake of 2 gs BSS daily. Although pre-treatment before infection and single daily dosing could represent a limitation of our findings to be reassessed in future experiments, BSS has the potential to undergo pharmaceutical development to be applied in chronic lung inflammatory diseases sustained by *P.aeruginosa* infection with huge amount of neutrophil infiltrates. Specific preclinical and clinical validation in CF lung models is required to test its application as a complementary drug to new generation mutant CFTR modulators.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Alice Rossi: Validation, Resources, Formal analysis, Writing – original draft. **Alessandra Bragonzi:** Conceptualization, Methodology, Resources, Formal analysis, Writing – original draft. **Melessike Medede:** Validation. **Ida De Fino:** Validation. **Giuseppe Lippi:** Writing – original draft. **Marco Prosdocimi:** Writing – original draft. **Anna Tamanini:** Conceptualization, Methodology. **Giulio Cabrini:** Conceptualization, Methodology, Writing – original draft. **Maria Cristina Dechecchi:** Conceptualization, Methodology, Writing – original draft.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcf.2022.08.005.

References

[1] Barry PJ, Taylor-Cousar JL. Triple combination cystic fibrosis transmembrane conductance regulator modulator therapy in the real world - opportunities and challenges. Curr Opin Pulm Med 2021;27(6):554–66.
[2] Gabillard-Lefort C, Casey M, Glasgow AMA, Boland F, Kerr O, Marron E, Lyons AM, Gunaratnam C, McElvaney NG, Reeves EP. Trikafta Rescues CFTR and Lowers Monocyte P2X7R-induced Inflammasome Activation in Cystic Fibrosis. Am J Respir Crit Care Med 2022;205(7):783–94.
[3] Ribeiro CMP, McElvaney NG, Cabrini G. Editorial: novel anti-inflammatory approaches for cystic fibrosis lung disease: identification of molecular targets and design of innovative therapies. Front Pharmacol 2021;12:794854.
[4] Cabrini G, Rimessi A, Borgatti M, Pinton P, Gambari R. Overview of CF lung pathophysiology. Curr Opin Pharmacol 2022;64:102214.
[5] Lampronti I, Dechecchi MC, Rimessi A, Bezzerri V, Nicolis E, Guerrini A, Tacchini M, Tamanini A, Munari S, D'Aversa E, Santangelo A, Lippi G, Sacchetti G, Pinton P, Gambari R, Agostini M. Cabrini G. β-sitosterol reduces the expression of chemotactic cytokine genes in cystic fibrosis bronchial epithelial cells. Front Pharmacol 2017;8:236.
[6] Marahatha R, Gyawali K, Sharma K, Gyawali N, Tandan P, Adhikari A, Timilsina G, Bhattarai S, Lamichhane G, Acharya A, Pathak I, Devkota HP, Parajuli N. Pharmacologic activities of phytosteroids in inflammatory diseases: mechanism of action and therapeutic potentials. Phytother Res 2021;35(9):5103–24.

[7] Wang J, Wu Q, Ding L, Song S, Li Y, Shi L, Wang T, Zhao D, Wang Z, Li X. Therapeutic Effects and molecular mechanisms of bioactive compounds against respiratory diseases: traditional chinese medicine theory and high-frequency use. Front Pharmacol 2021;12:734450.
[8] Facchini M, De Fino I, Riva C, Bragonzi A. Long term chronic Pseudomonas aeruginosa airway infection in mice. J Vis Exp 2014;85:51019–29.
[9] Bragonzi A. Murine models of acute and chronic lung infection with cystic fibrosis pathogens. Int J Med Microbiol 2010;300:584–93.
[10] Li H, Zhao X, Wang J, Dong Y, Meng S, Li R, Niu X. Deng X. β-sitosterol interacts with pneumolysin to prevent Streptococcus pneumoniae infection. Sci Rep 2015;5:17668–77.
[11] Reyes AWB, Arayan LT, Huy TXN, Vu SH, Min W, Hur J, Kim S. β-sitosterol contributes in the resistance to invasion and survival of Brucella abortus 544 within RAW264.7 cells, and cytokine production with reduced susceptibility to infection in BALB/c mice. J Microbiol Biotechnol 2020;30(4):482–489.
[12] Ding K, Tan YY, Ding Y, Fang Y, Yang X, Fang J, Xu DC, Zhang H, Lu W, Li M, Huang SC, Cai ML, Song Y, Ding YJ. Zhang SM. β-Sitosterol improves experimental colitis in mice with a target against pathogenic bacteria. J Cell Biochem 2010;120(4):5687–94.
[13] Lee JH, Lee JY, Park JH, Jung HS, Kim JS, Kang SS, Kim YS, Han Y. Immunoregulatory activity by daucosterol, a beta-sitosterol glycoside, induces protective Th1 immune response against disseminated Candidiasis in mice. Vaccine 2007;25(19):3834–40.
[14] Fraile L, Crisci E, Córdoba L, Navarro MA, Osada J, Montoya M. Immunomodulatory properties of beta-sitosterol in pig immune responses. Int Immunopharmacol 2012;13(3):316–21.
[15] Bouic PJ. Sterols and sterolins: new drugs for the immune system? Drug Discov Today 2002;7(14):775–8.
[16] Racette SB, Lin X, Lefevre M, Spearie CA, Most MM, Ma L, Ostlund RE Jr. Dose effects of dietary phytosterols on cholesterol metabolism: a controlled feeding study. Am J Clin Nutr 2010;91(1):32–8.