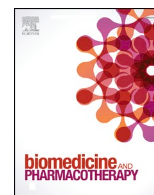
Biomedicine & Pharmacotherapy 146 (2022) 112525

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha



Reduction of oxidative stress blunts the NLRP3 inflammatory cascade in LPS stimulated human gingival fibroblasts and oral mucosal epithelial cells

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A R T I C L E I N F O

Keywords:
ROS
NLRP3
COX-2
5-LOX
Oral mucositis
Flavocoxid

A B S T R A C T

The therapeutic armamentarium for the treatment of oral mucositis is very poor. Catechin and baicalin are two natural flavonoids that have been individually reported to have a curative potential. Flavocoxid is a mixed extract containing baicalin and catechin showing antioxidant effects and anti-inflammatory activity mainly due to a dual inhibition of inducible cyclooxygenase (COX-2), 5-lipoxygenase (5-LOX) and NLRP3 pathway. The aim of this study was to evaluate the anti-inflammatory and anti-oxidant effects of flavocoxid in an "in vitro" model of oral mucositis induced by triggering an inflammatory phenotype in human gingival fibroblasts (GF) and human oral mucosal epithelial cells (EC).

GF and EC were challenged with lipopolysaccharide (LPS 2 μg/ml) alone or in combination with flavocoxid (32 μg/ml).

Flavocoxid increased Nrf2, prompted a marked reduction in malondialdehyde levels and reduced the expression of COX-2 and 5-LOX together with PGE_2, and LTB_4 levels. Flavocoxid caused also a great decrease in the expression of NF-κB and turned off NLRP3 inflammasome and its downstream effectors signal, as caspase-1, IL-1β and IL-18 in both GF and EC cells stimulated with LPS.

These results suggest a correlation between oxidative stress and NLRP3 activation and indicate that flavocoxid suppresses the inflammatory storm that accompanies oral mucositis. This preclinical evidence deserves to be confirmed in a clinical setting.

1. Introduction

Oral mucositis (OM) is an exaggerated inflammatory response with specifically clinical characteristics such as pain, erythematous lesions, ulcers, dysphagia and difficulty in food intake; this clinical condition impairs patient quality of life and limits the compliance to anticancer drugs [1–4]. The pathogenesis of mucositis is more complex than the historical view that described mucositis as a result of the non-specific direct effects of radiation or chemotherapy. Indeed, mucosal cells are more susceptible to the damage induced by ionizing radiation and chemotherapy because of their high turnover. Both therapies induce cell cycle arrest and boost the inflammatory cascade, triggering apoptosis with the consequent loss of highly dividing mucosal cells [5]. The cascade of events depicted as damage processing after radiation exposure starts with the initial induction of free radicals and reactive oxygen species (ROS); ROS release results in the modification of the activity of various transcription factors and activation of different intra- and intercellular signaling chains. The primary damage response, noted in the cells and tissues of the oral mucosa, is characterized by the expression of early response genes c-Jun, c-Fos and Egr-1, the activation of the transcription factors such as Nuclear Factor kappa B (NF-κB) and vascular adhesion molecules. This is followed by upregulation of genes that results in the production of pro-inflammatory cytokines such as interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor

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https://doi.org/10.1016/j.biopha.2021.112525
Received 6 October 2021; Received in revised form 30 November 2021; Accepted 8 December 2021
Available online 11 December 2021
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alpha (TNF-α) leading to apoptosis and tissue injury [6,7]. Moreover, myelosuppression increases Gram-negative organisms; consequently, bacterial cell wall products such as lipopolysaccharide (LPS) amplify mechanisms that exaggerate and extend the injury by stimulating infiltrating macrophages to produce additional damaging cytokines [8].

LPS leads to the activation of NF-κB and subsequent upregulation of NLRP3 inflammasome that has been shown to have a central role in several pathological conditions, including oral mucositis induced by radiation [9,10] and periodontitis [11,12]. Moreover, NLRP3 activation may be amplified by ROS and inflammatory lipid mediators such as Prostaglandin E_2 (PGE_2) [13]. NLRP3 inflammasome has three main components that synergistically assembly: NLRP3, apoptotic speck-containing protein (ASC) adaptor and pro-caspase 1. Following NLPR3 activation, pro-caspase 1 is transformed into caspase-1 by enzymatic cleavage and this enzyme allows the release of the mature forms of IL-1β and IL-18, two powerful inflammatory cytokines involved in OM.

However, despite the growing interest in the management of this disease, the available therapeutic intervention for the treatment of OM is very poor. Recently phyto-therapeutic compounds have been proposed as an alternative option in the management of this troublesome condition. Baicalin from *Scutellaria baicalensis* and catechin from *Acacia catechu* are two natural flavonoids that have been indirectly and anecdotally reported to have a curative potential in oral mucositis [14,15], but no clear proof-of-concept study is available in the scientific literature. Interestingly, both natural compounds have been previously proposed for the management of several pathological conditions including inflammation, viral and bacterial infection, cancer and cardiovascular disease [16–18]. Flavocoxid is a combination of the two flavonoids. Several experimental and clinical findings have pointed out the efficacy of this phyto-therapeutic agent in reducing the oxidative stress and the consequent inflammatory cascade in numerous models of acute and chronic inflammation [19–25]; flavocoxid has been also reported to act as a balanced inhibitor of cyclooxygenase 1/2 (COX-1/COX-2) and of 5-lipoxygenase (5-LOX). Indeed, prostaglandins play an important role in the development of oral mucositis [26]. More specifically, PGE_2 is significantly increased in the inflamed GF cells when compared to the GF with a normal phenotype [26]. In agreement with this finding non-steroidal anti-inflammatory drugs are effective in oral mucositis [27]. By contrast, the role of 5-LOX products is still not well understood [28], even if Leukotriene B4 (LTB_4) may have a reinforcing role in amplifying the inflammatory cascade triggered by ROS. Therefore, interrupting the correlation between ROS and inflammatory lipid mediators release and NLRP3 activation might be considered as a rational strategy to counteract the inflammatory response in OM.

In light of these observations, the aim of this study was to investigate the effects of flavocoxid, a balanced inhibitor of COX 1/2 and of 5-LOX with well-established anti-oxidant and anti-inflammatory activity, in an "in vitro" model of oral mucositis induced by triggering an inflammatory phenotype in human GF and EC cells.

2. Materials and methods

2.1. Cell Cultures

Human primary gingival fibroblasts (atcc-pcs-pcs201-018) and human oral mucosa epithelial cells (cticc1.8.3 sk0251) were obtained from LCC Standards S.r.l Milan, Italy and CliniSciences s.r.l. Rome, Italy, respectively. Cells were put in culture in a medium made by DMEM, 10% fetal calf serum, 1% antibiotic mixture and incubated at 37 °C with 5% of CO_2. The medium was changed with a time interval of 2 days and the cells were re-plated.

2.2. Treatments of cells

GF and EC cells were cultured in six well culture plates at a density of 2.5×10^5 cells/well and were challenged with LPS (2 μg/ml; Escherichia coli serotype 055:B5; Sigma-Aldrich, Milan, Italy) alone or with catechin, baicalin, flavocoxid (a flavonoid mixture of baicalin and catechin with a ratio of 4:1 Primus Inc. Scottsdale, AZ, USA) at the dose of 300 μg/ml, 65 μg/ml and 32 μg/ml respectively. Cells were harvested after 4 h of incubation with the treatments. LPS and treatments doses were chosen according to previously published papers [5,20,29–31].

2.3. MTT assay

Cell viability was evaluated by MTT assay. GF and EC cells were grown and then treated with LPS (2 μg/ml), LPS + catechin (300 μg/ml), LPS + baicalin (65 μg/ml) LPS + flavocoxid (32 μg/ml) when reached confluence. In particular, CTRL, LPS, LPS + catechin, LPS + baicalin and LPS + flavocoxid were tested in a 96-well plate at a density of 8×10^4 cells/well for 24 h to evaluate the cytotoxic effect. The tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma Aldrich, Milan, Italy) was dissolved in sterile filtered PBS, and 20 μl of the mixture were added into each well 5 h before the end of the 24 h of incubation. Medium was removed and the insoluble formazan crystals were dissolved with dimethyl sulfoxide (DMSO; 200 μl/well) following 5 h. The difference between the values obtained at 540 and 620 nm of absorbance was used to calculate the average of replicates and to evaluate cytotoxicity. Results were expressed as % of cell viability compared to untreated cells and reported as means and SD [31,32].

2.4. Measurements of cytokines by Enzyme-Linked Immunosorbent Assay (ELISA)

IL-6 levels were measured in the cell culture supernatants using an Enzyme-Linked Immunosorbent Assay (ELISA) kits (Abcam, Cambridge, UK or Thermo Fisher, Waltham, MA, USA), in agreement with the instructions reported by the manufacturer. All the samples were evaluated in duplicate and the obtained results were interpolated with the pertinent standard curves [33,34].

2.5. Real Time Quantitative PCR amplification (RTqPCR)

Total RNA was extracted from GF and EC cells for RTqPCR using Trizol LS Reagent (Invitrogen, Carlsbad, CA, US). Two μg of total RNA was reverse transcribed in a final volume of 20 μl using a Superscript VILO kit (Invitrogen). cDNA (1 μl) was added to the EvaGreen qPCR Master Mix (Biotium Inc., Fremont, CA, USA) (20 μl per well). The final primer concentration selected to perform the analysis was 10 μM. Samples were run in duplicate and β-actin was used as an endogenous control. Results were calculated using the $2^{-\Delta\Delta CT}$ method and expressed as n-fold increase in gene expression using the CTRL group as calibrator [35–37]. Primers used for targets and reference genes are listed in Table 1.

2.6. Malondialdehyde assay

The effects of the several treatments as antioxidants against lipid peroxidation in GF and EC cells were investigated on the basis of the level of malondialdehyde (MDA). 200 microliters of supernatant were

Table 1
Primer list.

Gene	Sequence
β-actin	Fw: 5'AGAGCTACGAGCTGCCTGAC3' Rw: 5'AGCACTGTGTTGGCGTACAG3'
COX-2	Fw: 5'GTTCCACCCGCAGTACAGAA3' Rw: 5'AGGGCTTCAGCATAAAGCGT3'
5-LOX	Fw: 5'TGGCGCGGTGGATTCATAC3' Rw: 5'AGGGGTCTGTTTTGTTGGCA3'

mixed with 800 μl of PBS, 25 μl of butylated hydroxytoluene (BHT), and 500 μl of Trichloroacetic acid (TCA). The mixture was vortexed and incubated on ice for 2 h. After centrifuging at 2000 g for 15 min, 1 ml of supernatant was taken out and transferred into tube containing 75 μl of 0.1 M EDTA and 250 μl of 0.05 M TBA. The tube was boiled in water bath for 15 min and then, left to cool at room temperature before read by spectrophotometer at 532 nm and 600 nm wavelengths. The result obtained was compared to standard curve [38,39].

2.7. Western Blot Analysis

After 4 h of incubation with the treatments cells were collected, and the protein expressions of NLRP3, p65, p-p65 and IL-1β (Abcam, Cambridge, UK), were evaluated, as previously described in detail [32].

2.8. Statistical analysis

Data are expressed as the mean ± standard deviation (SD) and the values reported are the results of three experiments. All assays were performed in duplicate to ensure reproducibility. The different groups were compared and analyzed using one-way ANOVA with Tukey post-test for comparison between the different groups. A p value < 0.05 was considered significant. Statistical analyses were performed using SPSS Statistics for Windows v22.0 (SPSS, Inc, Chicago, IL, USA). Graphs were prepared using GraphPad Prism (Version 8.0 for macOS, San Diego, CA, US).

3. Results

3.1. Flavocoxid does not affect cell viability

One hundred percent of viability was observed in control cells following 24 h incubation. The incubation with baicalin, catechin or flavocoxid did not affect GF and EC cells viability, thus demonstrating that these natural products do not have a cytotoxic effect and do not affect cell viability. Furthermore, LPS incubation did not change cell viability (Fig. 1).

3.2. Flavocoxid reduces oxidative stress

As a consequence of oxidative stress induced by LPS stimulation, a significant decrease in Nrf2 levels was observed in LPS-challenged cells ($p < 0.0001$ vs CTRL; Fig. 2). Baicalin and catechin treatment increased Nrf2 levels in GF and EC cells. The co-incubation with flavocoxid significantly improved the Nrf2 levels in the supernatants of GF and EC cells demonstrating a greater effect than that of baicalin and catechin alone ($p < 0.0001$ vs LPS; $p < 0.0001$ vs LPS + Baicalin; $p < 0.0001$ vs LPS + Catechin; Fig. 2).

To better examine the antioxidant effect of flavocoxid we measured the MDA levels in GF and EC cells. Control cells showed low levels of MDA, while LPS stimulation considerably increased MDA levels in both GF and EC cells ($p < 0.0001$ vs CTRL; Fig. 2). Baicalin and catechin treatment attenuated MDA production in GF and EC cells ($p < 0.0001$ vs LPS; Fig. 2). Co-incubation with flavocoxid resulted in a powerful reduction of MDA levels in gingival fibroblasts and oral mucosal epithelial cells demonstrating a greater effect than that of catechin and baicalin alone ($p < 0.0001$ vs LPS; $p < 0.0001$ vs LPS + Baicalin; $p < 0.0001$ vs LPS + Catechin; Fig. 2).

3.3. Flavocoxid blunts the NLRP3 cascade

LPS stimulation generated a marked induction in the protein expression of p-NFκB in GF and EC cells ($p < 0.0001$ vs CTRL; Fig. 3). Baicalin and catechin reduced the expression of p-NFκB in either GF and EC cells ($p < 0.0001$ vs LPS; Fig. 3). Flavocoxid markedly reverted the increase in the p-NF-κB protein levels in both GF and EC cells demonstrating that flavocoxid effect was greater than that of the two compounds alone ($p < 0.0001$ vs LPS; $p < 0.0001$ vs LPS + Baicalin; $p < 0.0001$ vs LPS + Catechin; Fig. 3).

LPS stimulation and the consequent NF-κB activation generated a marked increase in the protein expression of NLRP3 in both human GF and EC cells ($p < 0.0001$ vs CTRL; Fig. 4). Baicalin and catechin treatments reduced NLRP3 protein levels in either GF and EC cells ($p < 0.0001$vs LPS; Fig. 4). Flavocoxid co-incubation caused a more marked reduction in the expression of this inflammatory platform in

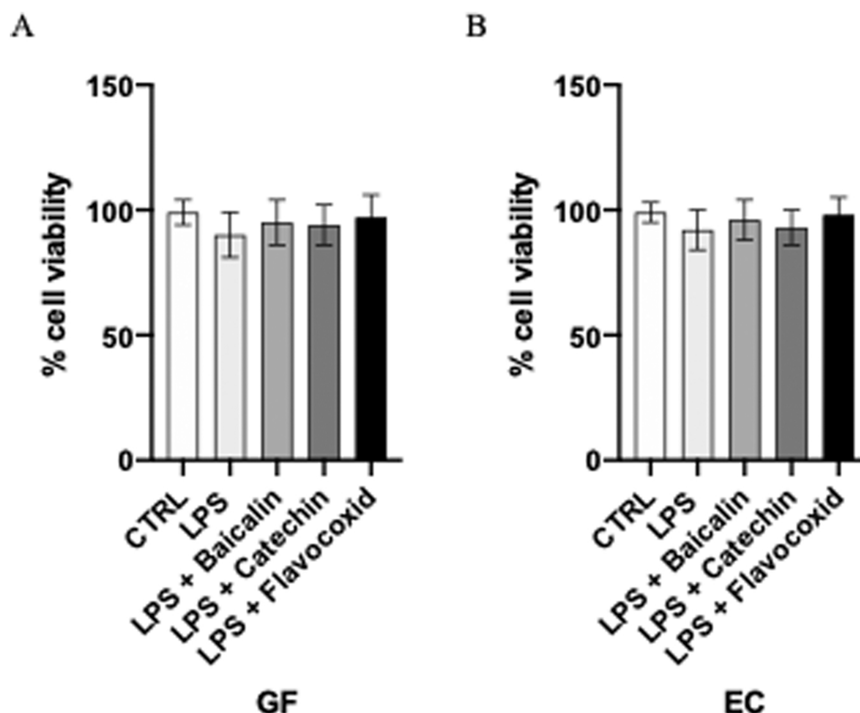

Fig. 1. The graphs show the cytotoxicity assay at 24 h in GF cells (A) and EC cells (B). Values are expressed as the means and SD.

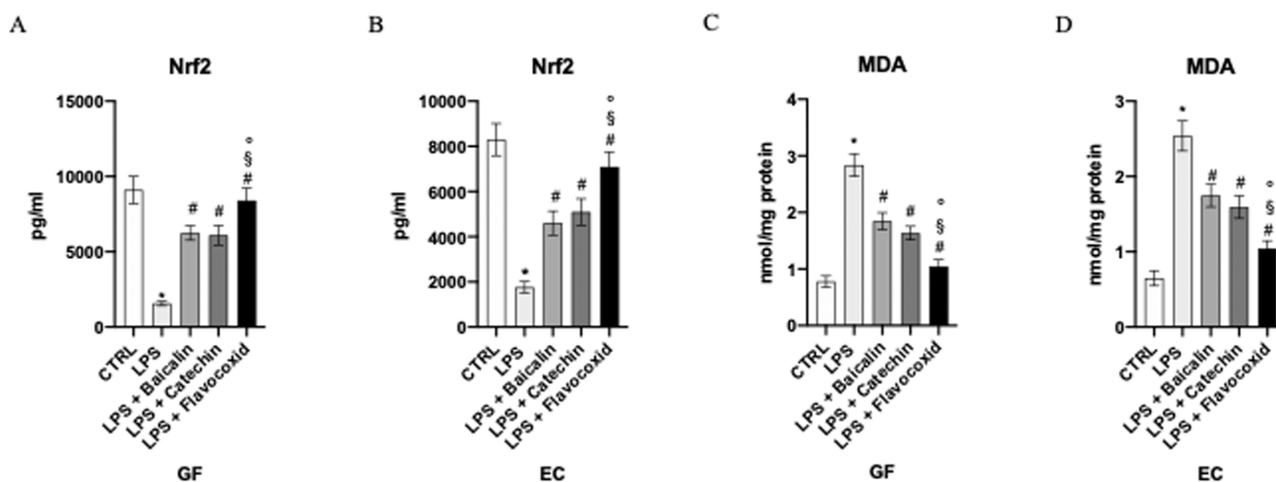

Fig. 2. The graphs represent the levels of Nrf2 (A), MDA (C) from GF cells and Nrf2 (B), MDA (D) levels from EC cells. Values are expressed as the means and SD. *$p < 0.0001$ vs CTRL; #$p < 0.0001$ vs LPS; §$p < 0.0001$ vs LPS + Baicalin; °$p < 0.0001$ vs LPS + Catechin.

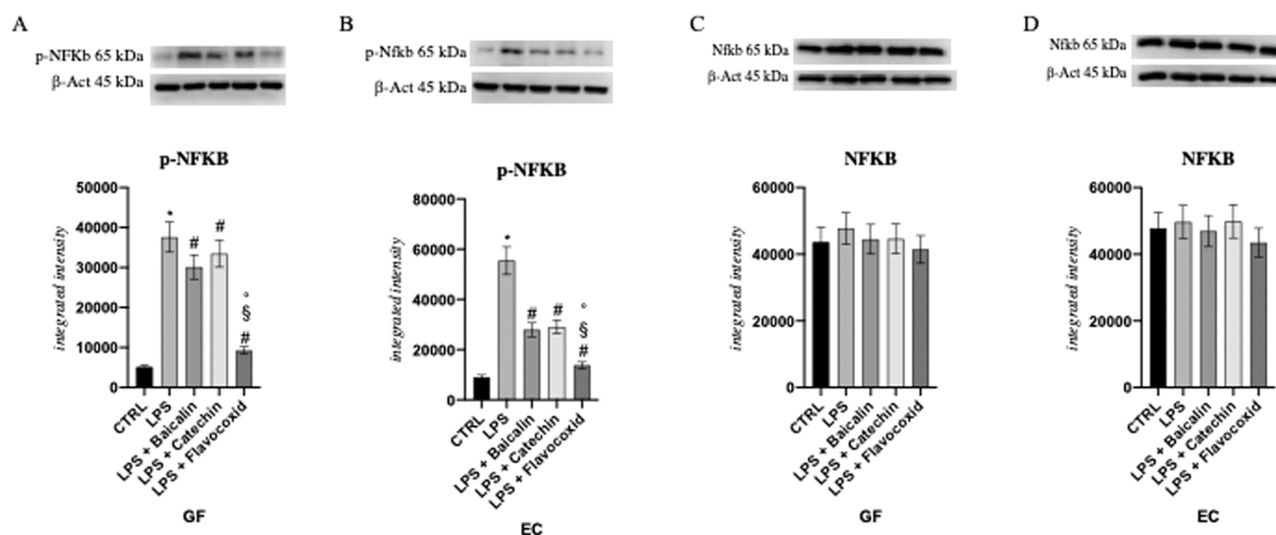

Fig. 3. The graphs represent the protein levels of p-NFkB (A), NFkB (C) from GF cells and p-NFkB (B) and NFkB (D) protein levels from EC cells. Values are expressed as the means and SD. *$p < 0.0001$ vs CTRL; #$p < 0.0001$ vs LPS; §$p < 0.0001$ vs LPS + Baicalin; °$p < 0.0001$ vs LPS + Catechin.

both GF and EC cells ($p < 0.0001$ vs LPS; $p < 0.0001$ vs LPS + Baicalin; $p < 0.0001$ vs LPS + Catechin; Fig. 4).

To explore the activation of the inflammatory cascade triggered by NLRP3, we evaluated the downstream signals and more specifically the protein expression of caspase-1, IL-β and IL-18 in human GF and EC cells challenged with LPS. The inflammatory stimulus prompted a marked production in the downstream products of the NLRP3 inflammasome in both GF and EC cells ($p < 0.0001$ vs CTRL; Fig. 5). Baicalin and catechin reduced caspase-1, IL-1β and IL-18 levels in both GF and EC cells ($p < 0.0001$vs LPS; Fig. 5). Flavocoxid more efficiently blunted the increase of caspase-1, IL-1β and IL-18 in the LPS stimulated GF and EC cells demonstrating a greater effect than that of catechin and baicalin alone in inhibiting the downstream products of the NLRP3 inflammasome ($p < 0.0001$ vs LPS; $p < 0.0001$ vs LPS + Baicalin; $p < 0.0001$ vs LPS + Catechin; Fig. 5). Moreover, to deeply investigate the effect of flavocoxid on NFkB downstream signaling, we evaluated the protein level of IL-6. LPS stimulation caused a significant increase of IL-6 levels in GF and EC cells ($p < 0.0001$ vs CTRL; Fig. 5). Baicalin and catechin reduced IL-6 levels in either GF and EC cells ($p < 0.0001$ vs LPS; Fig. 5). Flavocoxid markedly reverted the increase in the IL-6 protein levels in both GF and EC cells demonstrating that flavocoxid effect was greater than that of the two compounds alone ($p < 0.0001$ vs LPS; $p < 0.0001$ vs LPS + Baicalin; $p < 0.0001$ vs LPS + Catechin; Fig. 5).

3.4. Flavocoxid downregulates COX-2, 5 LOX expression and reduced PGE_2, LTB_4 levels

LPS challenge produced a marked stimulation of the inducible form of COX-2 in GF and EC cells ($p < 0.0001$ vs CTRL; Fig. 6). Baicalin and catechin treatments decreased the mRNA expression of COX-2 in either GF and EC cells ($p < 0.0001$ vs LPS; Fig. 6). Flavocoxid markedly reverted the increase in the message for COX-2 in both GF and EC cells demonstrating that the effect of flavocoxid was greater than that of the two compounds alone ($p < 0.0001$ vs LPS; $p < 0.0001$ vs LPS + Baicalin; $p < 0.0001$ vs LPS + Catechin; Fig. 6). 5-LOX was also marked stimulated by the LPS priming in both GF and EC cells ($p < 0.0001$ vs CTRL; Fig. 6). Baicalin and catechin treatments reduced 5-LOX expression in both GF and EC cells ($p < 0.0001$ vs LPS; Fig. 6). The natural product abolished the augmented message for 5-LOX in either human GF and EC cells showing a greater effect than that of catechin and baicalin alone ($p < 0.0001$ vs LPS; $p < 0.0001$ vs LPS + Baicalin; $p < 0.0001$ vs LPS + Catechin; Fig. 6).

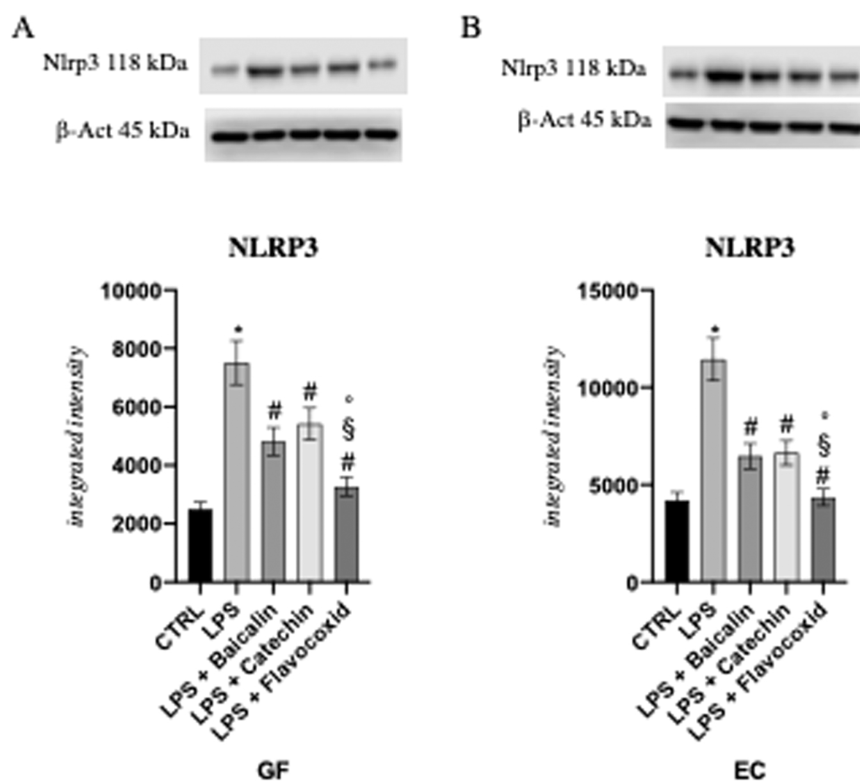

Fig. 4. The graphs represent the protein levels of NLRP3 from GF cells (A) and EC cells (B). Values are expressed as the means and SD. *$p < 0.0001$ vs CTRL; #$p < 0.0001$ vs LPS; §$p < 0.0001$ vs LPS + Baicalin; °$p < 0.0001$ vs LPS + Catechin.

The metabolites of COX-2 and 5-LOX were also markedly released in the supernatants of GF and EC cells upon LPS stimulation ($p < 0.0001$ vs CTRL; Fig. 7). Baicalin and catechin treatment decreased PGE_2 and LTB_4 levels in either GF and EC cells ($p < 0.0001$ vs LPS; Fig. 7). Co-incubation with flavocoxid resulted in a powerful reduction of both PGE_2 and LTB_4 levels in GF and EC cells demonstrating a greater effect than that of catechin and baicalin alone in inhibiting the release of COX-2 and 5-LOX metabolites ($p < 0.0001$ vs LPS; $p < 0.0001$ vs LPS + Baicalin; $p < 0.0001$ vs LPS + Catechin; Fig. 7).

4. Discussion

OM affects patients receiving antineoplastic drugs or radiation therapy. The pathogenesis of OM is still poorly understood and several molecular pathways are subjected to intense investigation with the aim to clarify the mechanism(s) underlying this troublesome clinical condition.

In the last few years the research's attention has been drawn to new inflammatory platforms, collectively known as inflammasomes [40]. Inflammasomes are multiprotein oligomers that, in response to inflammatory triggers, activate intracellular molecular signals cleaving the inactive precursor of IL-1β into the mature protein [40]. One of the most studied inflammasome is the NLRP3 inflammasome, consisting in NOD-like receptor NLRP3, the adapter protein termed "apoptosis-associated speck like protein" containing a caspase recruitment domain" and pro-caspase 1. In the recent years a two-signal model for NLRP3 inflammasomes activation has been proposed. The priming signal (signal 1) is provided by microbial components such as LPS, leading to the activation of the transcription factor NF-kB and subsequent upregulation of NLRP3. The activation signal (signal 2) is provided by a variety of stimuli including ROS that prompt the activation of NLRP3 inflammasome, causing the assembly of the three components and in turn producing the formation of the active form of caspase-1. Caspase-1 is then responsible for the production of the mature cytokines IL-1β and IL-18, previously stored as inactive molecules. NLRP3 activation might occur following an exaggerated production of PAMPs and ROS in oral mucositis, a common sequela in patients undergone to chemotherapy or radiation therapy [27], for this reason interrupting the correlation between inflammatory lipid mediators release, ROS and NLRP3 activation might be considered as a rational strategy to counteract the inflammatory response in OM.

Our results showed that flavocoxid increased Nrf2 levels, blunted the oxidative stress-induced inflammatory cascade triggered by LPS and turned off the NLRP3 inflammasome platform in oral mucositis. The experimental paradigm used in the present study slightly differs from a previously reported model [21] and we choose to reproduce an inflammatory phenotype in human GF and EC cells through LPS without the use of recombinant cytokines. Indeed, LPS stimulation results in a more robust activation of the inflammatory cascade and, from a translational point of view, more efficiently reproduces the clinical setting of oral mucositis since the pathogenesis of oral mucositis involves the participation of bacterial microorganisms.

The management of oral mucositis is not easy to implement, as shown by the evidence that keratinocyte growth factor-1 is the only compound approved by the US Food and Drug administration and by the European Medicine Agency for this unpleasant condition. In addition, this medicine may be used only in "at-high-risk" patients [41]. Phyto-therapeutic agents have been also proposed for the cure of oral mucositis [42]. In agreement with these scientific evidences, flavocoxid blunted the inflammatory phenotype triggered by LPS in GF and EC cells by limiting the activation of COX-2 and the production of PGE_2 and turned off the NLRP3 inflammatory platform due to its antioxidant activity that led to a marked reduction in NF-κB protein expression [20]. Flavocoxid is a combination of baicalin and catechin. Indeed, the two components of flavocoxid alone weakly exerted a low degree of protection in our experimental model, instead our results strongly suggested that the association of both, as in flavocoxid, markedly amplified and potentiated the inhibitory effect on the LPS induced inflammatory

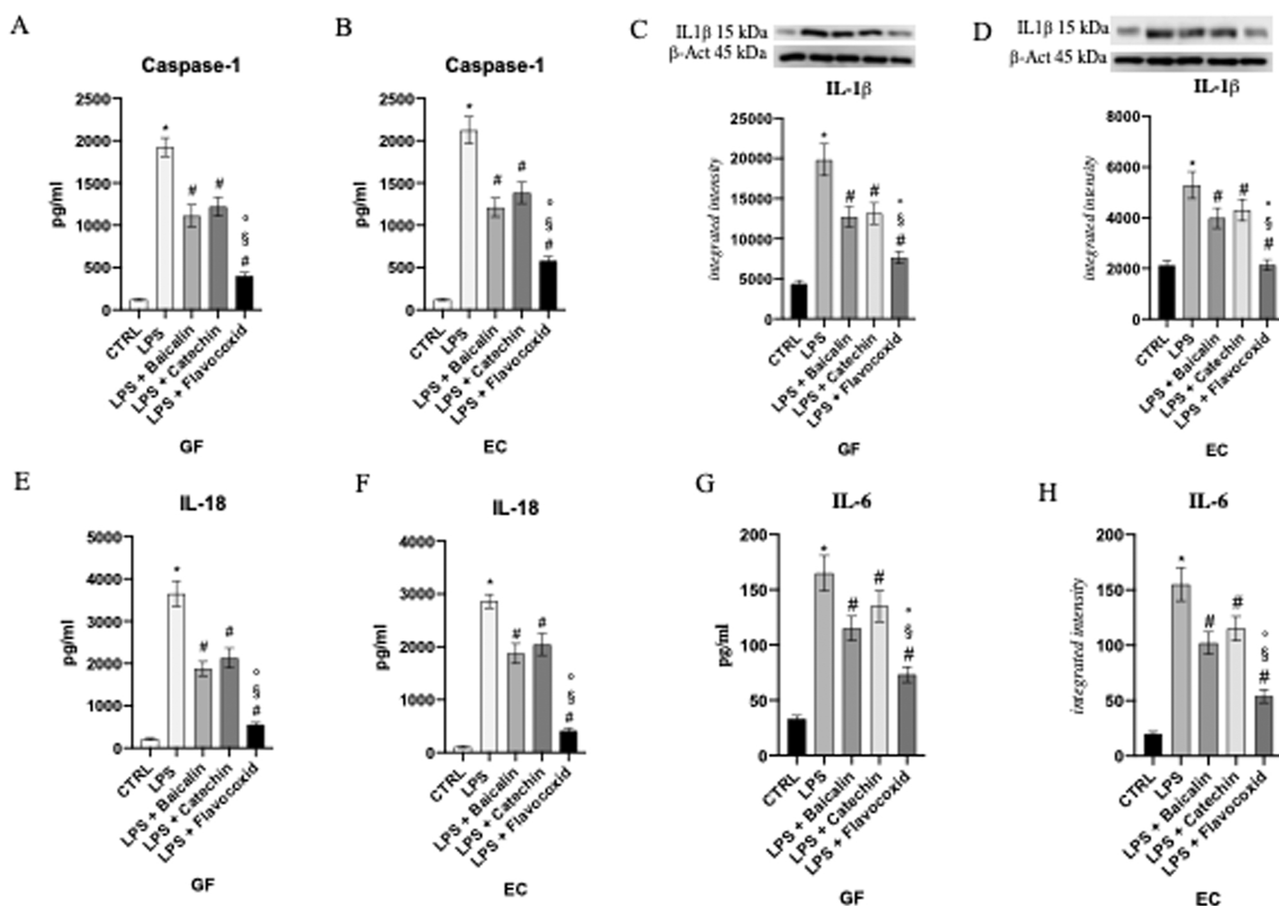

Fig. 5. The graphs represent protein levels of Caspase-1 (A), IL-1β (C), IL-18 (E), IL-6 (G) from GF cells and Caspase-1 (B), IL-1β (D), IL-18 (F) and IL-6 (H) protein levels from EC cells. Values are expressed as the means and SD. *p < 0.0001 vs CTRL; #p < 0.0001 vs LPS; §p < 0.0001 vs LPS + Baicalin; °p < 0.0001 vs LPS + Catechin.

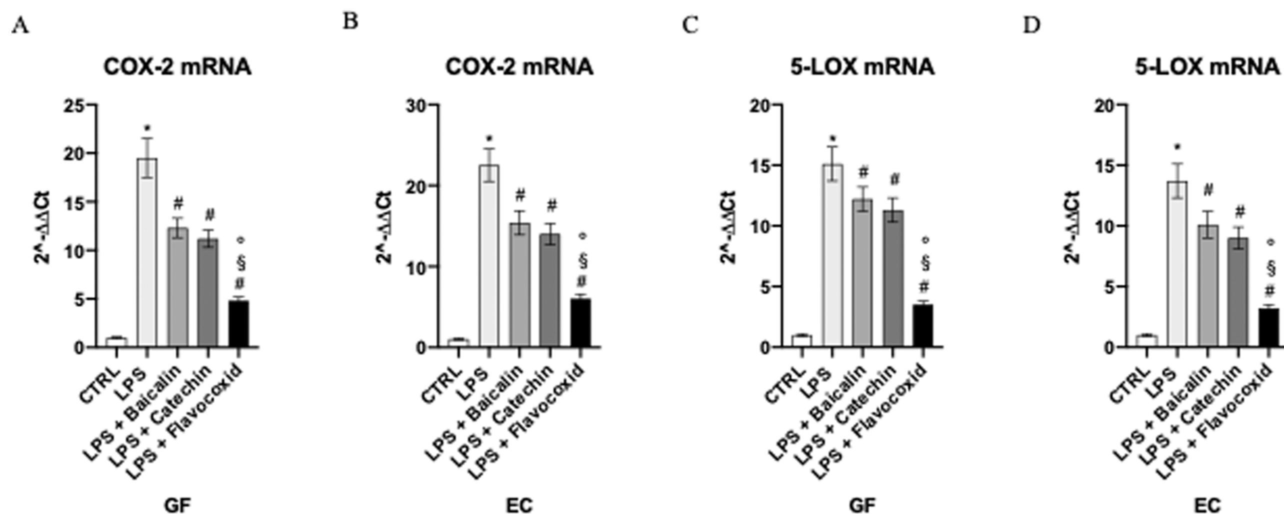

Fig. 6. The graphs represent qPCR results of COX-2(A), 5-LOX (c), mRNA expression from GF cells and COX-2 (B), 5-LOX (D) mRNA expression from EC cells. Values are expressed as the means and SD. *p < 0.0001 vs CTRL; #p < 0.0001 vs LPS; §p < 0.0001 vs LPS + Baicalin; °p < 0.0001 vs LPS + Catechin.

phenotype. This confirms that the "therapeutic strategy" of combination of the two natural products deserves attention.

Besides, our results suggest that NLRP3 inflammasome may represent a target to design rational curative strategies in OM and it could assume an important translational significance since it is deeply implicated not only in the pathogenesis of inflammatory conditions but also in traumatic and ischemic states [43–45]. Flavocoxid was able to inhibit NLRP3 inflammasome activation in an experimental model of Alzheimer's disease [46] and in the present model this therapeutic approach was able to inhibit NLRP3, thanks to its antioxidant activity, and consequently NLRP3 priming signal. In addition, flavocoxid directly inhibits COX-2 that reduces PGE_2, a prostaglandin that amplifies a

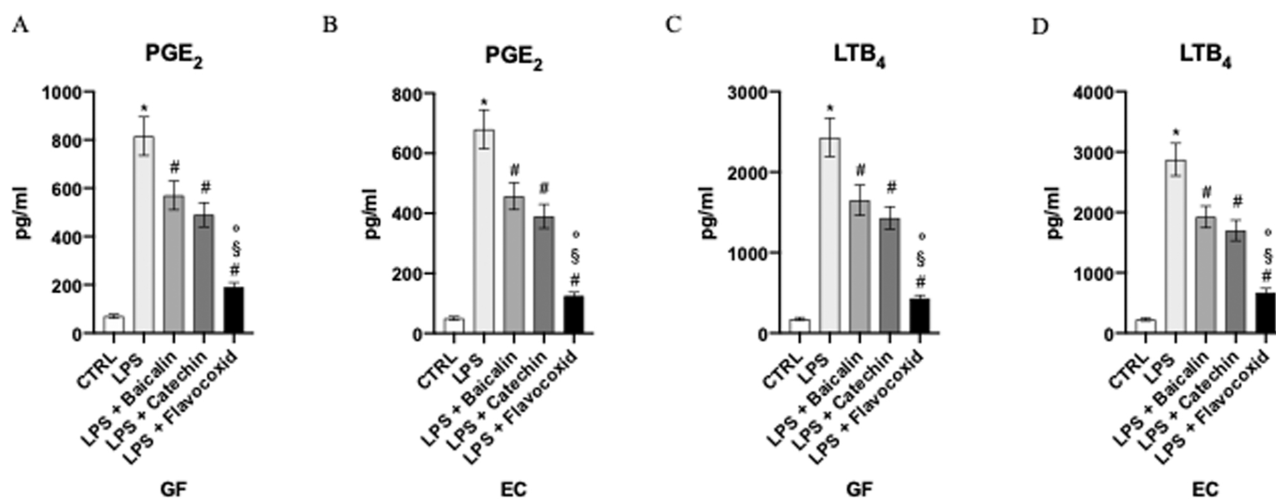

Fig. 7. The graphs represent the levels of PGE_2 (A), LTB_4 (C) from GF cells and PGE_2 (B), LTB_4 (D) levels from EC cells. Values are expressed as the means and SD. $^*p < 0.0001$ vs CTRL; $\#p < 0.0001$ vs LPS; $\S p < 0.0001$ vs LPS + Baicalin; $^\circ p < 0.0001$ vs LPS + Catechin.

widespread activation of this inflammasome platform. Our results suggested that flavocoxid also blocked 5-LOX, thus reducing LTB_4 release: this additional effect might have some theoretical advantages in patients with OM. The dampening in the production of the 5-LOX derived product might result either in a reduced ROS formation and in a blunted vasoconstriction that may concur to exacerbate the symptomology of oral mucositis. Therefore, flavocoxid might represent an attracting biomolecule for several reasons: a) it shows a strong antioxidant activity, as previously reported [25], increasing Nrf2 levels, preventing the generation of MDA and in turn inhibiting both NF-kB activation and 5-LOX activity; b) it is a compound that halts PGE_2 release, a mediator deeply involved in the inflammatory reactions of the oral cavity; c) it is a powerful inhibitor of the NLRP3 inflammasome that has a central role in the inflammatory cascade during oral mucositis; d) it is endowed of a high translational potential that may facilitate and speed up the transition from “bench to bed-site”.

This latter consideration is of paramount importance in the context of the bioassay that we used to investigate the potential benefit of flavocoxid. In fact, it can be argued that the present “in vitro” model of oral mucositis may represent an optimal methodology for those biomolecules or natural compounds which are already on the market and if found active in the pre-clinical screening, may be ready for a clinical evaluation, thus dramatically speeding up the time for regulatory approval. Indeed, isolated case reports have generated safety concerns on an association between flavocoxid and acute liver injury. If this evidence would be confirmed, the use of this medical food would be markedly limited and dampened, especially in the clinical setting of cancer patients undergoing chemotherapy. Indeed, a post-marketing surveillance study has ruled out this concern: in fact, the incidence rate of liver injury in 3337 flavocoxid users was low and not statically elevated compared to 6674 non-steroidal anti-inflammatory drugs recipients [47]. The safety of flavocoxid was also demonstrated in a recent clinical trial conduct in patients with Duchenne muscular dystrophy. Flavocoxid was well tolerated and no patients dropped out from the study [48].

In conclusion, our results indicate for the first time that flavocoxid efficiently suppresses the inflammatory storm that accompanies oral mucositis. These preclinical evidences deserve to be deeply investigated in patients suffering from OM.

CRediT authorship contribution statement

Conceptualization: **Giacomo Picciolo** and **Federica Mannino**. Formal analysis: **Natasha Irrera**, **Letteria Minutoli** and **Domenica Altavilla**. Funding acquisition: **Francesco Squadrito**; Methodology: **Mario Vaccaro** and **Giacomo Oteri**. Supervision: **Giovanni Pallio**. Writing – original draft: **Giacomo Picciolo** and **Federica Mannino**. Writing – review & editing: **Giovanni Pallio**.

Declaration

Funding

This work was supported by departmental funding assigned to Professor Francesco Squadrito. Flavocoxid was a kind gifts of Primus Inc. (Scottsdale, AZ, USA).

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

None.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2021.112525.

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