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Anti-Inflammatory effect of the Saffron Stigma and Saffron Petal Extract on LPS stimulated human Caco-2 cell by transductional signal modulation of FBW7/IKBα

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Abstract. Although the progression of IBD therapy is controlled with chemical drugs and biological therapies, healing results cannot yet be achieved, along with the inevitable side effects. As a result, a variety of research have focused on exploring novel therapies and found that natural products can serve as promising therapeutic agents for IBD through their anti-inflammatory and antioxidant effect. Recently, the chemical constituents of the main saffron processing bio-product, the petals of C. sativus, have attracted the attention of researchers. We compare the anti-inflammatory effect of the Saffron Stigma Extract (SSE), Saffon Petal Extract (SPE) and Petals/Stigma Extract (SPE/SSE) on lipopolysaccharide (LPS)-stimulated human Caco-2 cell monolayers by analyzing FBW7/ signaling IkBα, upstream of the activation of inducible molecules such as iNOS and COX-2. The results of this study provide further support for the possible use of SPE in medicine, raising awareness of the potential of the waste product generated in the production of the saffron spice. Compared to the SPE/SSE is not able to attenuate the pro-inflammatory response, and further investigations should be undertaken to understand what kind of negative interaction is triggered between the two components of the spice.

Keywords: IBD, inflammation, intestinal epithelial cells, saffron petals extract, saffron stigma extract.

1. INTRODUCTION

Crocus sativus L., commonly known as saffron, is, widely cultivated in many Mediterranean countries and parts of Asia (De Cecco, F et al., 2022). Recently, the chemical constituents of the main saffron processing by-product, the petals of C. sativus, have attracted the attention of researchers. In the flowering stage, both stigmas and petals of C. sativus contain various bioactive flavonoids, alkaloids, and coumarins. Although the stigmas and petals are similar in composition, there are differences in their metabolite content. Interestingly, a total of 147 flavonoid metabolites were detected in the stigmas and petals, of which 23 were significantly upregulated in the petals. Inflammatory bowel disease (IBD) is a chronic state of gastrointestinal inflammation and is mainly grouped into Crohn's disease (CD) and ulcerative colitis (UC). Although the pathophysiology of IBD are still unclear, high levels of pro-inflammatory cytokines are present in the gut of patients with IBD and have a crucial role in the dysfunction of mucosal homeostasis contributing to the pathogenesis of IBD (Chen Y, et al., 2021). In the present study, we compare the anti-inflammatory effect of the Saffron Stigma Extract (SSE), Saffon Petal Extract (SPE) and Petals/Stigma Extract (SPE/SSE) on lipopolysaccharide (LPS)-stimulated human Caco-2 cell monolayers analysing FBW7/IKBα signaling.

2. MATERIALS & METHODS

2.1 Cell culture

The human colon adenocarcinoma Caco-2 cell line (ATCC® TIB-202™ Rockville, MD, USA) was cultured as previously reported by (Wu XX, et al., 2019).

2.2 Cytotoxicity assay

The Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay (Sigma-Aldrich, St. Louis, MO, USA) was performed, as previously described (Franceschelli S, et al., 2016).

2.3 ROS Detection

An NBT (nitroblue tetrazolium) assay was performed as previously described to detect intracellular ROS levels (Franceschelli S, et al., 2019)

2.4 RNA extraction, reverse transcription, and Real-Time PCR

Cells were collected in 1mL QIAzol lysis reagent (Qiagen, Hilden, Germany), total RNA extraction and Real-Time PCR was performed as previously described (Patruno A, et al., 2015).

2.5 Western Blot Analysis

Western blot analysis was performed as described previously (Franceschelli S, et al., 2019) using the following antibodies against iNOS (OTI1E5; 1:700), IKBα (NFKBIA) (OTI1D4; 1:400), FBXW7 (OTI6B1; 1:1000), COX-2 (ab52237; 1:500), and β-actin (Santa Cruz Biotechnology).

2.6 Statistical analysis

Quantitative variables are summarized as the mean value and standard deviations (SD) in the Tables and Figures. To assess the accuracy of fold change data, the 95% confidence interval (95% CI) and standard error (SE) were determined. A Student's t-test for unpaired data was applied to evaluate the significance of differences. All tests were two-tailed. The threshold of statistical significance was set at p=0.05. Data analysis was performed on GraphPad Prism 6 Software, version 6.01, 2012.

3. RESULTS

3.1 Saffron Stigma Extract and Stigma/Petals Extract does not affect the viability of epithelial cells.

In order to determine the concentrations of SSE and of SPE/SSE which has nontoxic to cells, we examined the cell viability in Caco-2 cells after incubation with different concentrations of SSE (50ng/ml, 100ng/ml, 500 ng/ml, 1ug/ml, 5ug/ml, 10ug/ml, 25ug/ml, 50ug/ml, 100ug/ml, 250 ug/mL, 500ug/ml, 1mg/ml) and of SPE/SSE (we used petals and stigmas in the ratio of 2:1- range to 50 and 25 ng/ml from 1 and 0.5 mg/ml), for 24 h. MTT assay showed that SSE and SPE/SSE did not affect the viability of Caco-2 cells at concentrations lower than 1mg/ml and 1-0.5 mg/ml, respectively (Fig. 1A, B). Furthermore, the superoxide anion radical-scavenging activity was also measured in a non-enzymatic method at 24h. The generation of superoxide anions was markedly inhibited (~50%) from the concentration of 25 µg/ml of SSE and 50-25 ug/ml of SPE/SSE in respect to cells

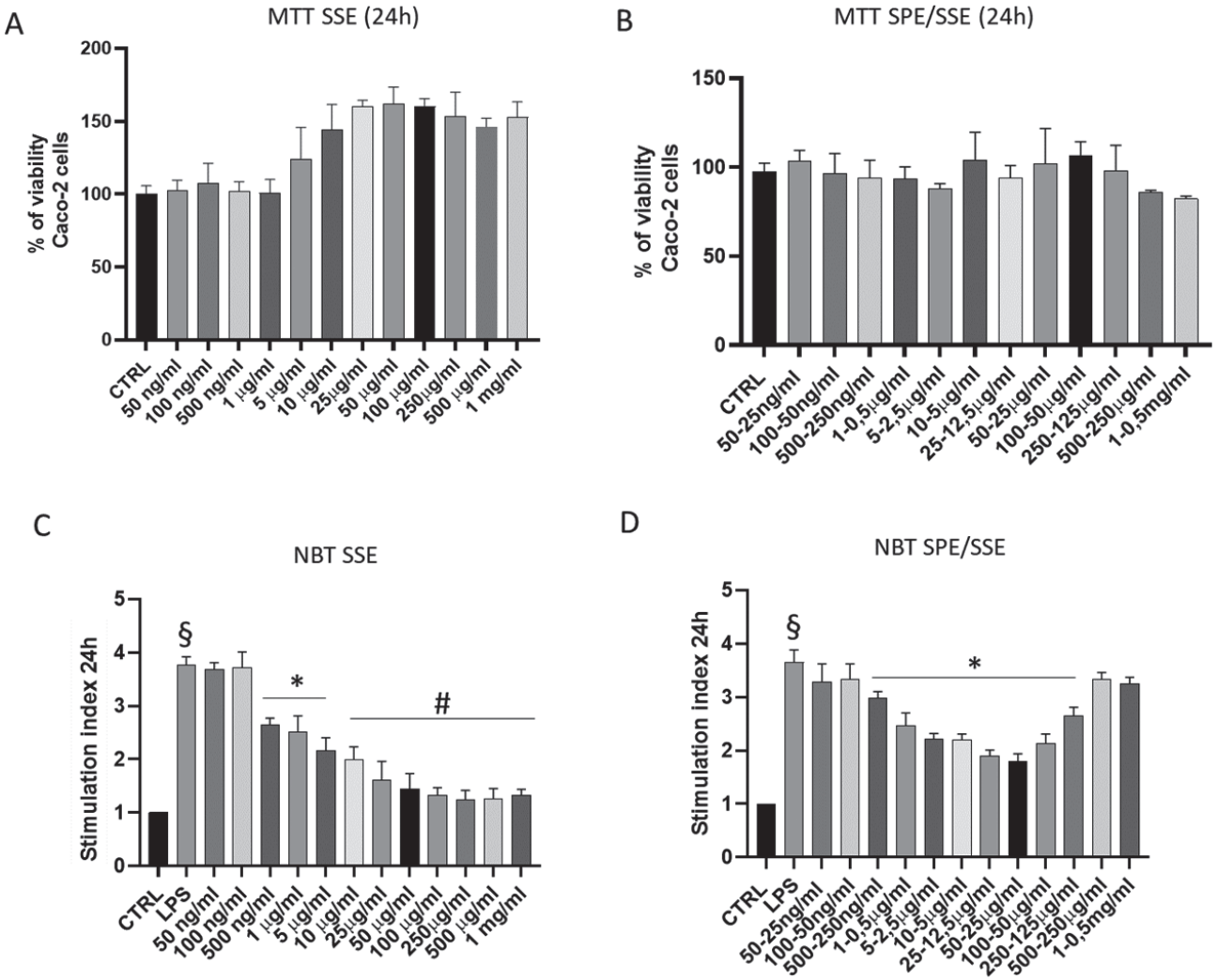

Figure 1. Cytotoxyc effect of SSE and SPE/SSE on Caco-2 cells. Cells were treated with SSE (A) and SPE/SSE (B) for 24h. Cells viability was measured by MTT assay as reported in Materials and Methods. Date are reported as % of viability in respect to control cells. Each bar represent means ±SEM (n=3); (C) Antioxidant activity of SSE (C) or SPE/SSE (D) against oxidative stress LPS-induced measured by NBT test. Results were registered as stimulation index (SI). SI value of 1 was assigned to control cells. Each bar represent means ±SEM (n=3). §p<0.005 vs CTRL; #p<0.05 and*p<0.01 vs LPS. (SSE: Saffron Stigma Extact; SPE/SSE:Petals/Stigma Extract; LPS: Lypopolysaccharide)

stimulated with LPS (Fig. 1C,1D). Of note, SPE/SSE at concentrations above 500-250 ug/ml loses its ability to neutralise superoxide anion. Thus, SSE at 25 µg/ml and SPE/SSE 50-25 ug/ml was more often used in the following experiments to test its activity against LPS-induced inflammation. SPE was used at a concentration of 50 ug/ml which, as per our previous results, is non-cytotoxic and induces significative reduction of the ROS production, as well as the expression of inducible iNOS and COX-2 (De Cecco, F et al., 2022).

3.2 Effect of Saffron Stigma, Saffron Petal and Petals/Stigma on inducible molecules

To compare the effects of SSE, SPE and SPE/SSE on epithelial cells, the expression of inducible proteins, known to be controlled by NF-kB (p65) and up-regulated in the inflammatory process, were checked. As shown in Figure 2, in Caco-2 cells exposed to LPS, SSE and SPE induce a down-regulation of both mRNA and protein expression of iNOS (Fig. 2A) and COX-2 (Fig. 2B) compared to activated cells. Instead, the combined extract of SPE/SSE, is not able to induce the down-regulation of the pro-inflammatory molecules analysed (Fig.2A,2B).

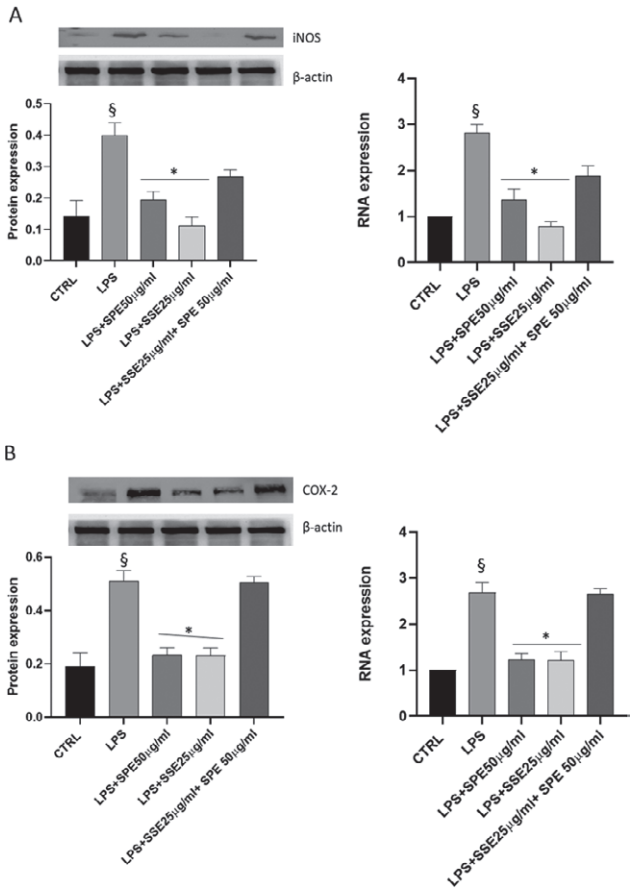

Figure 2. Effect of SPE, SSE, SPE/SSE on inducible molecules in Caco-2 cells. Representative image of Western blot analysis (left) with relative densitometry and real-Time PCR analysis (rigth) for iNOS (A), COX-2 (B). To the left, in the densitometric analysis (n = 3), each bar is reported as the intensity of optical density (IOD) ± SD. The following primer pair sequence was used: iNOS (F:5'- CATTGCTGTGCTCCATAGTTTC-3'-, R:5'- CAGGACGTAGTTCAGCATCTC-3'); COX-2 (F:5'-CGATGCTGTGGAGCTGTAT-3'; R:5'-CATTGCTGTGCTCCATAGTTTCG-3'); §P < 0.05, vs control cells and *P < 0.05 vs LPS-stimulated cells. (SSE: Saffron Stigma Extact; SPE: Saffron Petals Extract; SPE/SSE:Petals/Stigma Extract; LPS: Lypopolysaccharide)

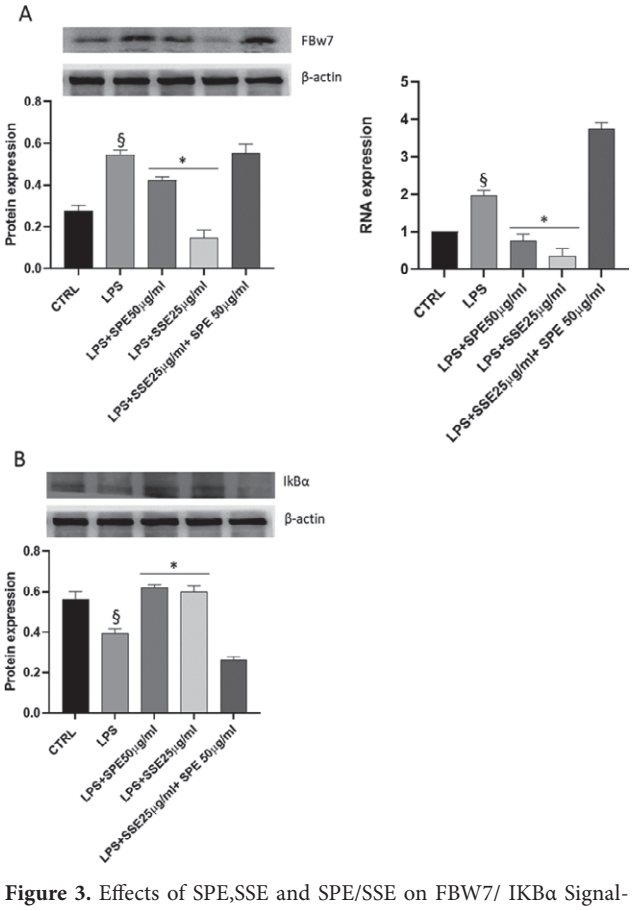

Figure 3. Effects of SPE,SSE and SPE/SSE on FBW7/ IKBα Signaling in Caco-2 cells. Representative image of Western blot analysis for FBW7 (A) and IKBα (B). To the left, in the densitometric analysis (n = 3), each bar is reported as the intensity of optical density (IOD) ± SD. §P < 0.05, significance *vs* control cells. For qRT-PCR analysis, the following primer pair sequence was used: FBW7 (F:5'-CAGTCCGCTGTGTTCAATATG-3', R:5'-GCCCTGTTAACGTGTGAATG-3'); 18S (F:5'- CTTTGCCATCACTGCCATTAAG -3', R:5'-TCCATCCTTTACATCCTTCTGTC-3'). *P < 0.05 significance *vs* LPS-stimulated cells.

3.3 Effect of Saffron Stigma and Petals on FBW7/IkBα signaling

Since in our previous paper (De Cecco, F et al., 2022) we have hypothesised that SPE exerts its regulatory effect on NF-kB interfering with FBW7, we studied and compared the effect of SPE with that of SSE and SPE/SSE. Expression levels of both mRNA and FBW7 protein were significantly up-regulated in cells stimulated with LPS confirming its role in regulating the inflammatory response (Figure 3A). Treatment with selected concentration of SPE and SSE induces a down-regulation of the expression of this ubiquitin which represents a negative regulator of the inflammatory process. At the same time, the protein levels of NF-kB inhibitor as IKBα were detected. Interestingly, IKBα was significantly expressed by SPE and SSE treatment compared to LPS-activated cells, leading us to hypothesize that both exert their regulatory effect on NF-kB by interfering with FBW7 (Figure 3B). However, the treatment of the activated intestinal epithelial cells with the SPE/SSE was unable to attenuate the inflammatory response induced by LPS and mediated by FBW7/NF-kB signaling.

4. DISCUSSION

Despite the incredible progress of modern medicine, significant obstacles remain in the treatment of IBD. There is high interest in alternative natural agents in the management of IBD because they are perceived to be safer than their synthetic equivalents due to their efficacy and minimal perceived side effects. Some herbs such as saffron, may control inflammation and improve cellular homeostasis in many diseases as well as peptic ulcer and UC (Singh G, et al., 2022). In our previous study we have demonstrated the efficacy of SPE as a protective agent against inflammation interfering with the FBW7 protein (De Cecco F, et al., 2022). In this study we compare the effect of SPE, SSE and a mix of SPE/SSE. There are several studies indicating that saffron and its constituents have an important role in inhibition the NF-κB signaling, but few studies have focused on comparing the effects of saffron and its components in cellular processes. (Zeinali M, et al., 2019). Our results showed that SPE can attenuate the over-expression of the inducible proteins iNOS and COX-2, in intestinal epithelial cells, in a similar way to SSE (Figure 2). Moreover, in this study, for the first time, we demonstrated that the SSE, as SPE, by reducing the expression of protein FBW7 inhibits the degradation of the IKBα subunit in intestinal epithelial cells (Figure 3B). This protein, by maintaining NF-kB in the inactive state, can alleviate a multitude of NF-kB-driven inflammatory diseases such as IBD. Finally, SPE/SSE treatment is not able to attenuate the pro-inflammatory response mediated by the regulation of FBW7/IKBα signalling.

This study confirmed that SSE and SPE possess anti-inflammatory and antioxidant activities, raising awareness of the potential of the waste product generated in the production of the saffron spice. However, the combined use of both is not able to attenuate the inflammatory response, therefore further investigations should be undertaken to understand what kind of negative interaction is triggered between the two components of the spice. The results of this study provide further insights into the study and confirmation of the effects of SPE in a two-dimensional coculture model to evaluate its possible use to support conventional IBD therapy through a clinical trial.

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