In vitro evaluation of anti-inflammatory activity of “Habanero” chili pepper (Capsicum chinense) seeds extracts pretreated with cellulase

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Research Article

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Abstract

The aim of this study was to explore the effect of capsaicin and particular phenolic compounds profile from cellulase assisted extracts of Habanero (*Capsicum chinense*) chili pepper seeds (CPS) on the concentration of cytokines (IL-2, IL-6, TNF-α, IL-1β) in murine macrophages (RAW 264.7) stimulated with lipopolysaccharides (LPS). Capsaicin was quantified by HPLC-DAD, and the phenolic profile was determined by UPLC-MS-QqQ. Anti-inflammatory activity was evaluated by Mouse Cytokine/Chemokine Magnetic Bead Panel 96-well plate assay. Among the 15 different phenolics found in CPS extracts obtained at 120 or 150 minutes of maceration with 2500 UI/L at 30ºC or 45 ºC in a 1:15 (w:v) proportion, the most abundant was vanillic acid (7.97–12.66 µg/g). The extract obtained at 30ºC and 120 minutes, showed similar effects than the observed for synthetic anti-inflammatory drugs indomethacin and dexamethasone and capsaicin standard. Beyond capsaicin, salicylic, protocatechuic and *trans*-cinnamic acids as well as vanillin in CPS extracts were correlated with the anti-inflammatory effect. On the other hand, capsaicin and chlorogenic acid contents were potential immunostimulants whose concentration varied depending on the cellulase treatment time.

1. Introduction

Capsaicin has been described as an efficient anti-inflammatory compound [1]. It links with transient receptor potential vanilloid (TRPV-1) and constrains the discharge of inflammatory mediators, including nitric oxide (NO) and pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and interleukin (IL)-6, in lipopolysaccharide (LPS)-stimulated human cells [2]. In general, chili pepper waste is an important source of compounds with interesting biological activities for pharmaceutical purposes [3]. In addition to capsaicin, fatty acids, and a variety of phenolic compounds and their glycosides are present in chili pepper seeds (CPS) extracts [4]. Glycosides of luteolin and quercetin obtained from chili pepper showed antioxidant and anti-inflammatory activities by inhibiting the release of TNF-α and IL-6 [5]. Acquavia et al. (2021), demonstrated the presence of quercetin and several of its derivates in “Habanero” extracts and their bioactivity [6]. Additionally, Pappalardo et al. (2021) proved liposomes as delivery system for white “Habanero” chili extracts to reduce cell viability of leukemia and other tumoral cells, and decrease the inflammation response in *in vitro* models [7].

Capsaicin also showed extensively anti-inflammatory and neuroprotective activities in LPS induced cell cultures, decreasing nitric oxide synthesis and TNF-α and IL-1β expression and blocking the protein expressions of NO synthase and cyclooxygenase-(COX)-2 [8]. Capsaicin and its analogues inhibited mRNA expression of IL-6, macrophage-inflammatory protein-3α (CCL20) and monocyte chemoattractant protein-1 (MCP-1) [9]. It also overturned T-cell activation and the advancement of cell division to S phase due to the attenuation of IL-2, interferon (IFN)- γ, IL-4 and IL-5 production. Additionally, in *in vivo* models, capsaicin decreased the plasma level of TNF-α and IL-6 and augmented the concentration of the anti-inflammatory IL-10 [10].

Cortés-Ferré et al. (2022) demonstrated that Enzyme Assisted Extraction (EAE) allowed the recovery of compounds with anti-inflammatory activity from CPS using water as solvent. Although significant differences were observed in the CPS extracts obtained at different times, temperature and cellulase concentration, no statistically significant correlation was found between capsaicinoids or total phenolic content (TPC) with the NO synthesis [11].

For this purpose, the aim of this study was to investigate the effect of capsaicin and particular phenolic compounds from cellulase assisted extracts of Habanero (*Capsicum chinense*) CPS on the concentration of inflammatory cytokines (IL-2, IL-6, TNF-α, IL-1β) in murine macrophages (RAW 264.7) stimulated with lipopolysaccharides (LPS).

2. Materials And Methods

2.1 Materials

Habanero chili (*Capsicum chinense*) seeds (CPS) were obtained from Italmesa Company, Puebla, Mexico. Celuzyme (Cellulase, 121,000 UI/kg) was kindly donated by Enmex S.A. de C.V., Estado de Mexico, Mexico. Capsaicin (> 97%, M2028) used as standard in the HPLC quantification, HPLC grade water and methanol, and analytical grade formic acid were purchased from Sigma (St. Louis, MO, USA). The solvents used for the UPLC-MS analysis were MS grade and purchased from Sigma-Aldrich (St. Louis, MO, USA). 60 phenolic standards were obtained for quantification as is described in Juarez-Trujillo et al. (2018) and all the stock solutions, samples, solvents and reagents were filtered through 0.20 µm PTFE membrane filters (Phenomenex, USA) before injection in the analytical platform [12]. For the *in vitro* experiments Dulbecco's Modified Eagle Medium (DMEM; Gibco, Gaithersburg, MD, USA), fetal bovine serum (FBS; GenDEPOT, Barker, TX, USA) and penicillin-streptomycin antibiotics were purchased. Finally, murine macrophage cell line (RAW 264.7) was obtained from American Type Cell Collection (ATCC, Manassas, VA, USA).

2.2 Methods

2.2.1 Enzymatic pre-treatment

Habanero seeds were mixed with the enzyme solution (2500 UI/L) in a 1:15 (w:v) proportion and incubated at a controlled temperature water bath. Enzymatic pre-treatment was performed at 30 ºC (T1) or 45 ºC (T2) and samples were obtained at 120 or 150 minutes to compare the effect of time and temperature. To study the effect of enzymatic pre-treatment time, a kinetic was performed at 30 ºC for 150 minutes (taking a sample every 30 minutes). The supernatant was recovered by filtration using a cloth and stored at -20 ºC until use.

2.2.2 Capsaicin quantification

The identification and quantification of capsaicin were performed according to the methodology of Othman et al. [13]. Briefly, after extraction from chili pepper seeds, the supernatant was filtered through a

0.45 µm nylon filter into a HPLC sample vial using a disposable syringe. The chromatographic analysis of capsaicin (CAP) and dihydrocapsaicin (DHC) was performed using high-performance liquid chromatography (HPLC) with DAD (Agilent 1100 Series Santa Clara, CA, USA) with a Zorbax Eclipse Plus C_{18} (particle size 4.6 x 100 mm 3.5µm) column at 40°C, a flow of 0.8 mL/min, and injection volume of 10 µL. The mobile phase consisted of (A) Water with 0.1% of formic acid and (B) Methanol. Chromatograms were obtained at l = 280 nm. The CAP and DHC concentrations in samples were expressed as µg of capsaicin equivalents/mL of extract.

2.2.3 Phenolic compounds composition and quantification

The identification and quantification of phenolic compounds was performed as previously reported in Juárez-Trujillo et al. [12]. Briefly, filtered samples were placed in 2 mL UPLC vials and the chromatography was performed in an Ultra High Performance Liquid Chromatograph (UPLC, Agilent 1290 series) coupled to a triple quadrupole mass spectrometer (MS-MS, Agilent 6460). The mobile phases were water with 0.1% of formic acid (A) and acetonitrile with 0.1% formic acid (B), at a flow of 0.3 mL/min and 2 mL of each sample were injected in the system. The column was an Eclipse Plus C18, 2.1 x 50 mm, 1.8 µm (Agilent Technologies, Santa Clara, CA, USA) used at a temperature of 40 ºC. Mass spectrometer operated in positive and negative electrospray ionization modes with the desolvation temperature of 300°C, the cone gas (N_2) flow of 5 L/min, the nebulizer pressure of 45 psi, the sheath gas temperature of 250°C, the sheath gas flow of 11 L/min, the capillary voltage (positive and negative) was 3,500 V and the nozzle voltage (positive and negative) of 500 V. The data was processed with the MassHunter Workstation Software version B.06.00 (Agilent Technologies) and the results are expressed as the average ± standard deviation of µg/g of extract and µg/mL for correlation analysis.

2.2.4 Evaluation of the Anti-Inflammatory Activity of CPS extracts

Murine macrophage cell line (RAW 264.7) was maintained in DMEM with 5% FBS and 1% penicillin/streptomycin at 37 ºC in a humidified atmosphere containing 5% CO_2. For the bioassay, $1x10^4$ cells/well were inoculated into 96-well plates using 100 µL of cell suspension. After 24 h incubation at 37°C, the cells were pretreated with 50 µL of CPS extracts (previously diluted in cell culture media at 1%) or capsaicin standard (CAPstd) or indomethacin or dexamethasone (1 µg/mL) for 48 h before adding 50 µL of growth media (for control wells) or 50 µL LPS (10 µg/mL). After 24 h, 100 µL the supernatant of LPS treated cells was used to evaluate the expression of IL-1β, IL-2, IL-6, and TNF-α using a custom Mouse Cytokine/Chemokine Magnetic Bead Panel 96-well plate assay (MCYTOMAG-70k, Merck Millipore, Boston, MA, USA). The antibody beads were prepared following the instructions specified in the kit. The cytokine concentrations were expressed as pg/mL.

2.2.5 Statistical Analyses

Data were expressed as means ± standard deviation of at least three independent experiments unless otherwise indicated. All variables were analyzed using ANOVA followed by a post hoc Tukey multiple

comparison test ($p < 0.05$). A multivariate principal components analysis of ($p < 0.05$) and Spearman ρ non-parametric correlation analysis were also performed using JMP 16.0 (SAS Institute Inc., Cary, NC, USA).

3. Results And Discussion

3.1 Phenolic compounds profile and quantification

The most abundant phenolic compound found in the extracts was vanillic acid (7.97–12.66 µg/mg), but no significant effects of time or temperature of extraction were observed (Table 1). Kim et al. after analyzed the methanolic extracts of 11 chili pepper varieties found out that the main phenolic compounds in the extracts from the fruits were p-hydroxybenzoic, vanillic, p-coumaric, ferulic and sinapic acids [14]. Similarly, Dos Anjos et al. found that on Brazilian chili pepper (*Capsicum frutescens*) pericarp one of the main phenolic compounds was vanillic acid (1.9 µg/mg) [15].

Table 1
Concentration of different phenolic compounds in aqueous extracts obtained from 30ºC or 45ºC and 120 or 150 minutes of cellulase treated chili pepper seeds.

Phenolic Compound [µg/mg]	Treatment			
	T1_120	T1_150	T2_120	T2_150
Precursors				
L-Phenylalanine	5.74 ± 0.79[a]	4.90 ± 0.27[a]	9.31 ± 1.33[a]	7.16 ± 0.39[a]
Phenolic acids				
4-Coumaric acid	0.67 ± 0.12[a]	0.48 ± 0.11[a]	0.98 ± 0.33[a]	0.80 ± 0.41[a]
4-hydroxybenzoic acid	1.72 ± 0.05[a]	1.49 ± 0.20[a]	2.11 ± 0.29[a]	2.02 ± 0.24[a]
Caffeic acid	0.05 ± 0.01[a]	0.04 ± 0.01[b]	0.10 ± 0.03[a]	ND
Chlorogenic acid	0.22 ± 0.03[a]	0.06 ± 0.02[c]	0.18 ± 0.06[b]	ND
Ferulic acid	1.62 ± 0.01[a]	1.13 ± 0.22[a]	1.95 ± 0.58[a]	1.51 ± 0.47[a]
Gentisic acid	0.15 ± 0.03[a]	0.11 ± 0.04[a]	0.21 ± 0.06[a]	0.16 ± 0.02[a]
Protocatechuic acid	1.07 ± 0.01[a]	1.11 ± 0.10[a]	1.53 ± 0.29[a]	1.33 ± 0.03[a]
Salicylic acid	0.24 ± 0.03[a,b]	0.18 ± 0.05[b]	0.28 ± 0.05[a,b]	0.31 ± 0.03[a]
Sinapic acid	0.34 ± 0.00[a]	0.25 ± 0.01[a]	0.37 ± 0.14[a]	0.22 ± 0.10[a]
trans-Cinnamic acid	0.11 ± 0.02[a]	0.08 ± 0.02[a]	0.13 ± 0.02[a]	0.14 ± 0.01[a]
Vanillic acid	9.97 ± 1.06[a]	7.97 ± 0.41[a]	12.66 ± 2.07[a]	12.58 ± 2.58[a]
Coumarins and lignans				
Umbelliferone	0.05 ± 0.00[a]	0.03 ± 0.00[a]	0.05 ± 0.01[a]	0.05 ± 0.00[a]
Vanillin	0.82 ± 0.02[a]	0.67 ± 0.00[a]	1.06 ± 0.15[a]	0.98 ± 0.12[a]
Flavonoids				
Quercitrin	0.49 ± 0.07[a]	0.69 ± 0.50[a]	0.84 ± 0.08[a]	0.55 ± 0.21[a]
[a]Different letters indicate significant differences in each panel after Tukey statistical test ($p < 0.05$).				
ND = Not Determined.				

Phenylalanin (phenolics' precursor) was the second most abundant compound, ranging from 4.90 to 9.31 µg/mg followed by protocatechuic, ferulic and 4-hydroxybenzoic acids (1.07 to 2.11 µg/mg). These compounds were not affected by temperature or time of extraction. The concentration of salicylic acid was higher after 150 minutes of cellulase treatment at 45 ºC (0.31 µg/mg) than the observed at 30 ºC (0.18 µg/mg). Surendran et al. demonstrated that the presence of salicylic acid and its derivates inhibited cellulose activity and therefore it affected its release from the cellular matrix [16].

Chlorogenic acid content was affected by the extraction time with the highest yield at 30 ºC and 120 minutes of enzyme exposure (0.22 µg/mg) that contrasted to the obtained at 150 minutes (0.06 µg/mg). Although not significant difference related with interaction of time and temperature was observed, temperature affected vanillin concentration, reaching higher contents at 45 ºC (0.98–1.06µg/mg) than the observed at 30 ºC (0.67–0.82 µg/mg). Finally, caffeic acid content was higher in the extracts obtained at 120 minutes (0.05–0.10 µg/mg). As with chlorogenic acid, caffeic acid concentration was lower or not detected in the CPS treated with cellulase for 150 minutes. Pettinato et al. showed that after 60 minutes of extraction, the concentration of caffeic acid decreased increasing that of its derivates [17].

3.2 Anti-inflammatory activity of CPS extracts

The CPS extracts obtained at 30 ºC and 150 minutes or at 45 ºC and 120 minutes decreased significantly ($p < 0.05$) the expression of IL-1β (Fig. 1a), a pro-inflammatory cytokine related with pain and diseases related with metabolic syndrome, sclerosis and rheumatoid arthritis [18], to levels like those obtained with dexamethasone (4.76 pg/mL) or indomethacin (4.93 pg/mL). Only the CPS extracts obtained at 30 ºC for 120 minutes (CAP = 0.30 µg/mL) had a moderately higher expression of this interleukin than capsaicin at 1 µg/mL but still had a lower concentration than the obtained for LPS control. All the treatments down-regulated the expression of IL-2 without significant differences among the CPS extracts or standards used as control (Fig. 1b). This is not just an immune and inflammatory cytokine but also a neuromodulation marker that activates the itchy response in nervous system [19], if the expression of IL-2 is reduced the itchy response related with capsaicin is reduced as well. For IL-6, an important implication cytokine related with not just acute pathological pain but also with chronic diseases and cancer, the CPS extracts obtained at 30 ºC for 120 minutes did not show significantly different levels than the obtained for LPS control (Fig. 1c). The expression of IL-6 in cells treated with CPS extracts didn't show significant difference between them (6946.60–8512.35 pg/mL) or with synthetic anti-inflammatory drugs (4828.43–5961.49 pg/mL) or capsaicin standard (5246.46 pg/mL). TNF-α expression was completely inhibited by dexamethasone and no significant differences were observed in the concentrations obtained with capsaicin, indomethacin and treatments performed at 30ºC (Fig. 1d). In contrast, the CPS extracts obtained at 45 ºC, and 120 minutes did not show significant difference in the concentration of this cytokine compared to LPS control. Concluding that CPS extract obtained at 30 ºC for 150 minutes showed the best results over the under-expression of all 4 cytokines tested, leading us to analyze the cytokine expression over time of extraction (kinetics).

Similarly with our results, Hudita et al. (2021) showed a significant decrease in IL-1β, IL-6 and TNF-α using alginate-encapsulated capsaicin (1.77 mg/mL). Bok et al. demonstrated that the administration of

peritoneal capsaicin reduced the expression of cytokines IL-1β and IL-6 after surgery injury in animal models [21]. Cho et al., studied the phenolic content of chili pepper fruits and their bioactivity over RAW 264.7 macrophages finding that the extracts rich in phenolics and flavonoids decreased significantly the expression of IL-6 and TNF-α in dose-dependent manner [5], concordant with our results. Malagarie-Cazenave et al. found that capsaicin standard (10 μM) reduce the expression of IL-6 and TNF-α in PC-3 cells of human prostate cancer [22]. We demonstrated that CPS extracts with reduced capsaicin content (CAP = 0.98 μM) decreased the over-expression of these cytokines and therefore their bioactivity was also related with phenolic compounds. Some authors relate the under-expression of TNF-α to the homeostasis of tissues after an inflammatory response from injuries, regulation of inflammation of adipocytes in chronic diseases, and with the glycemic control, insulin resistance and other ailments related with type 2 diabetes [19, 23, 24].

The concentration of most phenolic compounds explained 46.8% of the variation, while cytokine expression mainly affected the principal component 2 (Fig. 2a). TNF-α was positively correlated with capsaicin and caffeic acid concentration in the CPS extracts (Fig. 2b and 2c). IL-2 expression was positively related with chlorogenic acid concentration (Fig. 2d). These positive correlations indicate that extracts capsaicin, caffeic acid and chlorogenic acid at the concentration tested in these experiments were not anti-inflammatory but may act as immunostimulants. The administration of 1.8mg/kg of intraperitoneal capsaicin attenuated cellular immunosuppression [25]. Caffeic acid (10mg/kg) improved immune response in animal models targeting cytokines as TNF-α and IL-6 [26]. Chlorogenic acid loaded microcapsules (35mg/kg) reduced tumor growth by enhancing T-cell labor and by consequence inhibiting the immunosuppressive factor [27].

The expression of IL-6 was negatively related with the concentration of protocatechuic (Fig. 2e), salicylic (Fig. 2f), and trans-cinnamic acids (Fig. 2g) as well as with vanillin (Fig. 2h). Although no statistically significant differences were observed in the concentration of these compounds in the extracts, except for salicylic acid, these small changes generated an important effect on cytokines expression.

3.3 Changes in the anti-inflammatory activity of CPS extracts obtained at different times of enzymatic treatment

Among the four cytokines evaluated with CPS extracts obtained at 30 ºC and different times of enzymatic treatment, only IL-6 did not show significant differences (Fig. 3). For the IL-1β, the extracts obtained at 90 and 120 minutes were less anti-inflammatory than the obtained at 150 minutes (Fig. 3a). The extract at 120 minutes contained a higher amount of chlorogenic acid (0.22 μg/g) than the obtained at 150 minutes (0.06 μg/g) and the immunostimulant effect of this compound was clearly observed in the changes of concentration of IL-2 (Fig. 3b). The results of Spearman ρ test ($p < 0.05$) exhibited a positive correlation between the overexpression of this interleukin and the chlorogenic acid concentration in the CPS extracts. TNF-α showed a positive correlation with capsaicin concentration (Fig. 3d). Based on the kinetics results, the CPS extracts obtained in less than 90 minutes of cellulase treatment reached their

maximum inhibitory effect over the cytokines related with the inflammatory process. After 90 minutes, CPS extracts obtained from cellulase treatment may be used as immunostimulant.

4. Conclusions

We demonstrated that the CPS extracts obtained after cellulase treatment produced an anti-inflammatory effect like indomethacin, dexamethasone and capsaicin standard. The CPS extract obtained at 30ºC for 150 minutes reduced the concentration of IL-1b, IL-2, IL-6, and TNF-α in RAW264.7 cells stimulated with LPS. Based on the kinetic of enzyme assisted extraction, less than 90 minutes were enough to recover the anti-inflammatory compounds since potential immunostimulants were released after this time. Among the most anti-inflammatory compounds found in CPS extracts were salicylic, protocatechuic and trans-cinnamic acids along with vanillin that synergistically interacted with capsaicinoids. Further experimentation is required to evaluate the immunostimulant effect observed in CPS extracts obtained at 120 and 150 minutes of cellulase treatment and confirm the role of capsaicin, chlorogenic and caffeic acids on this bioactivity.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

All of the material is owned by the authors and/or no permissions are required.

Author contributions

H.E.C.F. prepared the first version of the manuscript and performed most of the experimental work. M.M.A. and M.A.R. performed an initial analysis of phenolic compounds, cytokines quantification and reviewed the last version of the manuscript. J.A.G.A and J.L.M.V. identified and quantified the phenolic compounds and reviewed the last version of the manuscript. J.A.G.U. designed and supervised the experiment and generated the last version of the manuscript.

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Declaration of competing interest

The authors confirm that they have no conflict of interest with respect to the work described in this manuscript.

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Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

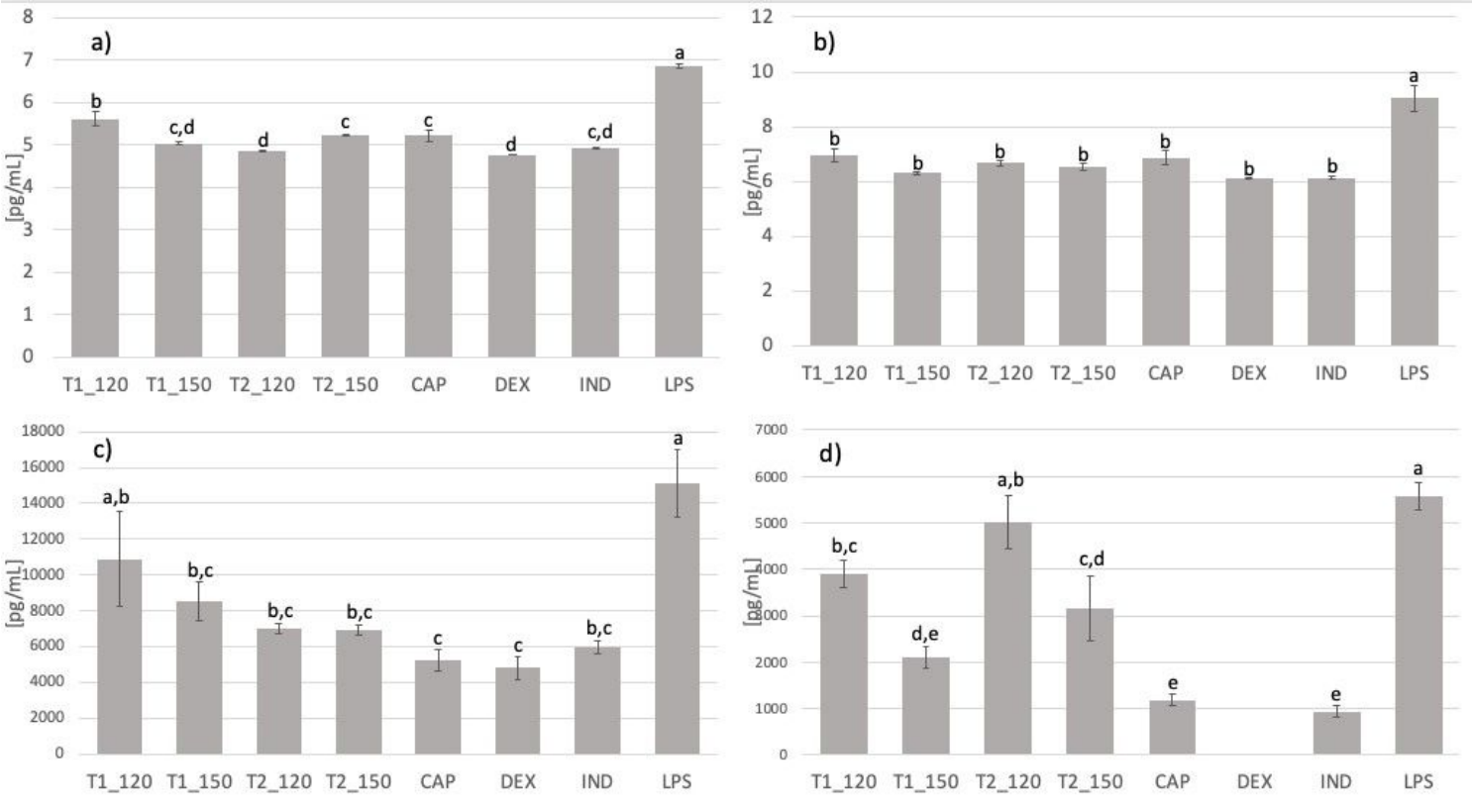

Figure 1

Cytokine expression [a) IL-1β, b) IL-2, c) IL-6 and d) TNF-α] of different enzyme assisted extracts of chili pepper seeds (T1_120= 30ºC/120min, T1_150= 30ºC/150min, T2_120= 45ºC/120min, T2_150=

45ºC/150min), Capsaicin (CAP), Dexamethasone (DEX), or Indomethacin (IND) compared with non-treated LPS-stimulated cells.

[a]Different letters indicate significant differences in each panel after Tukey statistical test ($p<0.05$).

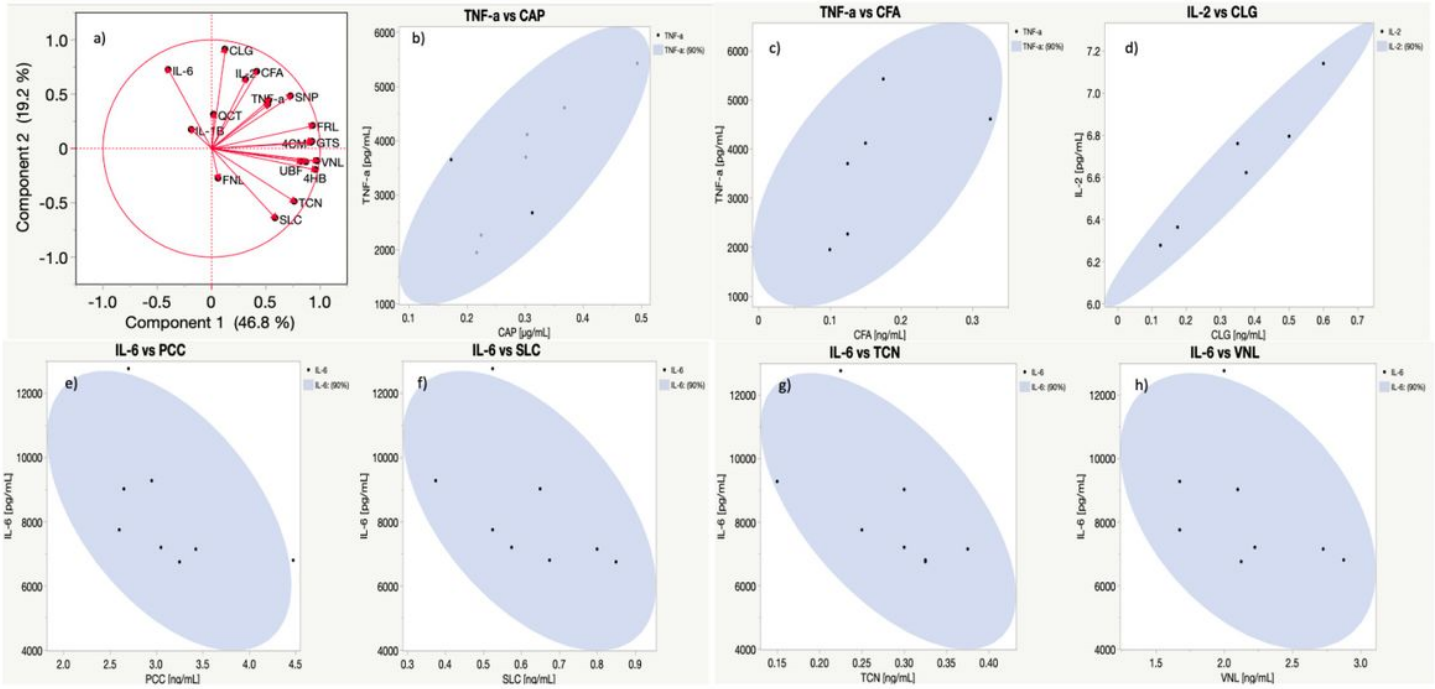

Figure 2

Principal components analysis (a) and correlation ellipse graphs of TNF-α vs CAP (b), TNF-α vs CFA (c), IL-2 vs CLG (d), IL-6 vs PCC (e), IL-6 vs SLC (f), IL-6 vs TCN (g), and IL-6 vs VNL (h). FNL: L-phenylalanine, PCC: protocatechuic acid, GTS: gentisic acid, 4HB: 4-hydroxybenzoic acid, VNA: vanillic acid, CLG: chlorogenic acid, CFA: caffeic acid, VNL: vanillin, 4CM: 4-coumaric acid, UBF: umbelliferone, FRL: ferulic acid, SNP: sinapic acid, SLC: salicylic acid, QCT: quercitrin, TCN: *trans*-Cinnamic acid, CAP: capsaicin.

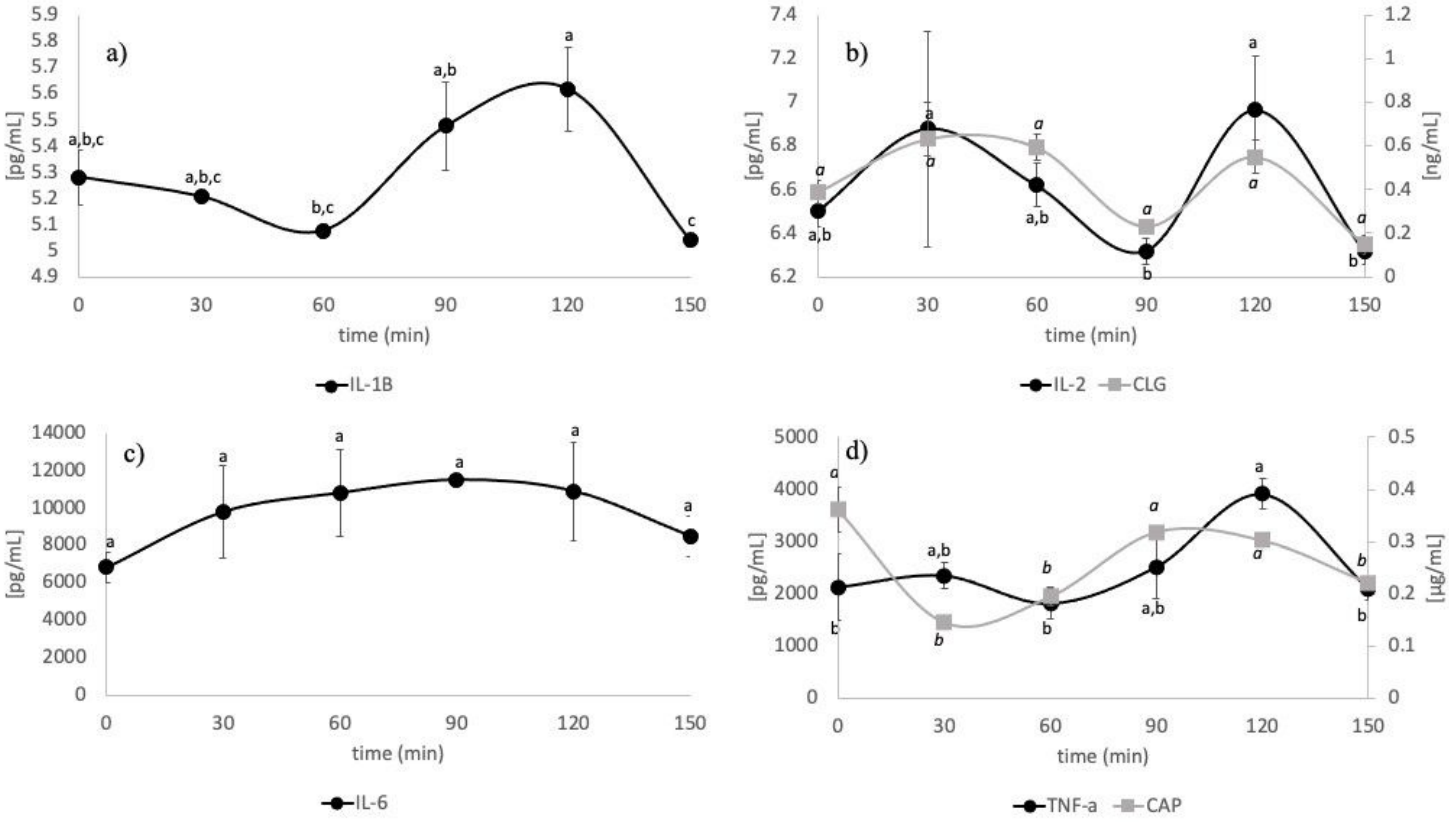

Figure 3

Cytokine expression over the extraction time at 30°C: a) IL-1β, b) IL-2 (main axis) / Chlorogenic acid concentration (secondary axis), c) IL-6, and d) TNF-α (main axis) / capsaicin concentration (secondary axis).

[a]Different letters indicate significant differences in each panel after Tukey statistical test ($p<0.05$).