Green extraction technologies and kombucha elaboration using strawberry tree (*Arbutus unedo*) fruits to obtain antioxidant and anti-inflammatory fractions

María de las Nieves Siles-Sánchez[1,2], Eva Tejedor-Calvo[3], Laura Jaime[1,2], Susana Santoyo[1,2], Diego Morales[4]*

[1]Sección Departamental de Ciencias de la Alimentación. Departamento de Química Física Aplicada. Facultad de Ciencias. Universidad Autónoma de Madrid (CEI UAM+CSIC), 28049, Madrid, Spain.

[2]Department of Production and Characterization of Novel Foods, Institute of Food Science Research—CIAL (UAM + CSIC), C/Nicolas Cabrera 9, Campus de Cantoblanco, Universidad Autónoma de Madrid, 28049 Madrid, Spain

[3]Laboratory for Aroma Analysis and Enology, Aragón Institute of Engineering Research (I3A), Department of Analytical Chemistry, Faculty of Sciences, University of Zaragoza, 50009 Zaragoza, Spain

[4]Departmental Section of Galenic Pharmacy and Food Technology, Veterinary Faculty, Avda. Puerta del Hierro s/n, Complutense University of Madrid, 28040, Madrid, Spain

*Corresponding author: diego.morales@ucm.es / diegomoraleshdz@gmail.com ; https://orcid.org/0000-0002-4600-6673

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Abstract

The fruits of *Arbutus unedo* (strawberry tree, ST) have been widely used for culinary and medicinal purposes because of their content in phenolic compounds and other bioactives. The aim of this study was to apply green technologies using water as solvent to obtain phenolic-rich fractions from ST fruits. Moreover, a kombucha beverage (KOM) using these fruits as substrate was prepared. Conventional (cold and hot water extractions) and advanced (ultrasound- and microwave-assisted-extractions) protocols were carried out, leading to fractions with high total phenolic content (TPC), up to 40.72 mg/100 mL in a microwave extract (MHWE), with also 7.89 mg total carbohydrates/mL and 106.12 µg soluble proteins/mL. Moreover, the kombucha showed even higher TPC (70.99 mg/100 mL). Biological activities were also tested for all the fractions, registering the highest antioxidant activity for KOM (2.63 and 6.22 µmol/mL of TEAC value as DPPH$^{\bullet}$ scavenger and cellular antioxidant activity, respectively) and MHWE (2.76 µmol/mL of TEAC value). Strong correlations were observed between TPC or specific compounds (mainly catechin derivatives) and antioxidant activity. Furthermore, KOM and the aqueous extracts were able to reduce pro-inflammatory cytokines (TNF-α, IL-1β, IL-6) in a cellular model.

Keywords: Microwave-assisted extraction; ultrasound-assisted extraction; fermentation; radical scavenging; cytokines; phenolic compounds

Abbreviations: ST (Strawberry tree); SCOBY/SC (Symbiotic Culture of Bacteria and Yeasts); UAE (Ultrasound-assisted extraction); MAE (Microwave-assisted extraction); TPC (Total phenolics content); TCC (Total carbohydrate content); CAA (Cellular antioxidant activity); TEAC (Trolox equivalent antioxidant capacity); CWE (Cold water extract); HWE (Hot water extract); UCWE (Ultrasound-assisted cold water extract); MHWE (Microwave-assisted hot water extract); KOM (Kombucha).

1. Introduction

A remarkable increase in chronic diseases incidence has been registered during the last decades, being oxidative stress and inflammatory processes identified as relevant risk factors. This situation has encouraged the development of novel approaches that may contribute to prevent and/or alleviate the progression of these non-communicable diseases. Since many existing therapeutic treatments caused side and adverse effects, important efforts are being dedicated to search and test antioxidant and immune-modulatory compounds and ingredients present in food and natural sources (Hajat & Stein, 2018; Ibarz-Blanch et al., 2023).

Arbutus unedo, commonly named 'strawberry tree' (ST) is an evergreen and flowering small tree or shrub, identified as a Mediterranean native plant that can be found in Southern, Western and Central Europe, Ireland, Canary Islands, North-Eastern Africa, and Western Asia (Morales, 2022; Tenuta et al., 2019). Their fruits have attracted relevant gastronomic interest, not only consumed as fresh berries, but also being traditionally used to prepare fermented and distilled beverages, jams, vinegars, and sauces. Regarding their composition, they usually show high carbohydrate contents (70-80% dry weight (dw))) with also high fibre percentage (10-30% dw) and interesting amounts of plant lipids (2-3% dw) and proteins (1-9% dw) (Ait Lhaj et al., 2021; Morgado et al., 2018). Besides these macronutrients, the presence of bioactive molecules in ST fruits deserves particular attention, highlighting their content of phenolic compounds, widely studied in this species, exerting interesting biological activities such as antioxidant (Zitouni et al., 2020) and immune-modulatory/anti-inflammatory, although this latter bioactivity was mainly studied in other ST components such as leaves and honeys (Moualek et al., 2016; Osés et al., 2020). Among the frequently targeted compounds, arbutin must be highlighted. This glycosylated form of hydroquinone can be found in ST and, together with the abovementioned activities, cosmetic industry pointed arbutin as a promising skin-whitening agent to correct blemishes and dark sports since it acts in the melanogenesis pathway inhibiting tyrosinase. Although the arbutin form commonly used for this purpose is synthetic, natural sources such as ST might be considered for the extraction and isolation of this molecule (Rudeekulthamrong & Kaulpiboon, 2020).

To obtain phenolics-rich fractions from ST fruits, different techniques have been used and can be found in the related literature. Most of them are based on conventional methodologies, mainly maceration or solid-liquid extractions using water or organic solvents (Masmoudi et al., 2020; Salem et al., 2018). Just a few works tested advanced technologies such as ultrasound- and microwave-assisted extractions (UAE and MAE, respectively) (Albuquerque et al., 2018; Boulanouar et al., 2013). In this sense, the potential of these methods is significantly underexploited, since the application of ultrasounds or microwaves allows the use of water and other GRAS solvents through protocols that are environmentally friendly and aligned with the principles of Green Chemistry. Moreover, many disadvantages related to conventional extractions are avoided with these methods, leading to higher extraction yields and shorter times and lower volumes of solvent, energy costs and residues generation, among other pros (Abdussalam-Mohammed et al., 2020).

Another interesting product that has experienced a remarkable increase in its acceptation and popularity in Western countries during the last years is kombucha. This beverage was originated in China around 200 B.C., traditionally prepared with tea leaves and sugar by the fermentative action of a consortium of yeasts and bacteria called 'SCOBY' (Symbiotic Consortium of Bacteria and Yeasts) (Diez-Ozaeta & Astiazaran, 2022; Morales, 2020). Together with tea or even replacing this material, different alternative substrates have been recently tested to design novel kombuchas with attractive organoleptic characteristics and interesting technological and functional properties (Freitas et al., 2022; Morales et al., 2023). As a matter of fact, a strawberry tree kombucha was elaborated previously, and the fermentation process was studied testing different SCOBYs, attending to pH, carbohydrate, protein and total phenolic compounds content, aromatic profile, etc. However, biological activities of the obtained kombuchas had not been evaluated yet (Tejedor-Calvo & Morales, 2023).

Thus, the purpose of the present work was to generate ST fruit kombucha and aqueous extracts applying conventional protocols (cold and hot water extractions) and also advanced technologies (ultrasound- and microwave-assisted extractions). The obtained preparations were characterized in terms of total carbohydrate, soluble proteins and total phenolics contents, also identifying and quantifying the specific phenolic compounds that were present in the samples. Finally, antioxidant capacity was assessed using the DPPH• radical scavenging assay and cellular antioxidant activity experiments, whereas anti-inflammatory activity was recorded attending to the modulation of cytokines secretion in a cellular model. Up to our knowledge, this is the first time that ST kombucha was studied in terms of its biological activities, and the first study that evaluated the cellular antioxidant activity of ST extracts to validate their ability to pass through cell membranes and reach their targets. This research was designed to confirm whether these novel ST fractions exerted more potent effects than conventional extracts.

2. Materials and methods

2.1. Biological material

ST fruits at the same stage of ripening were collected from trees located in Plasencia (Extremadura, Western Spain), freeze-dried (LyoAlfa 15-85 lyophilizer, Telstar, Terrasa, Spain), ground, mixed and sieved until a particle size < 0.5 mm was obtained. Powdered ST fruits were kept at -20 ºC until further use. The SCOBY (SC) used for the ST kombucha elaboration was kindly provided by a local producer and was previously analysed and utilized in a previous work related to ST kombucha (Tejedor-Calvo & Morales, 2023).

2.2. Reagents

Methanol (HPLC grade) was acquired from LAB-SCAN (Gliwice, Poland). Sodium carbonate (Na_2CO_3) and sulfuric acid (H_2SO_4) were purchased from Panreac (Barcelona, Spain). Bovine serum albumin (BSA), HCl (37%), phenol, Folin-Ciocalteu's phenol reagent, D-glucose, gallic acid, 2-2-diphenyl-1-pircrylhydrazyl (DPPH•), Trolox, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), 2,2′-azobis(2-amidinopropane) dihydrochloride (ABAP), 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), phorbol 12-myristate 13-acetate (PMA), and lipopolysaccharide from E. coli 055:B5 (LPS) were acquired from Sigma-Aldrich Química (Madrid, Spain). Bradford reagent was obtained from Bio-Rad (Hercules, CA, USA). Acetonitrile (HPLC grade), dimethyl sulfoxide (DMSO) (HPLC grade) and formic acid (purity ≥ 99 %) were from Macron Chemicals Fine (Gliwice, Poland). Chromatographic analytical reference standards (purity ≥ 95 %) such as catechin, gallic acid, and 3,4-dihydroxybenzoic acid were from Sigma-Aldrich Química (Madrid, Spain). Oenin chloride and arbutin were purchased in Extrasynthese S.A. (Genay, France) and TCI (Zwijndrecht, Belgium), respectively.

2.3. ST kombucha elaboration

The ST kombucha was prepared (in duplicate) according to the protocol described by Tejedor-Calvo and Morales (2023) (Tejedor-Calvo & Morales, 2023), with slight modifications. Briefly, lyophilized ST powder (12 g/L) and white cane sugar (70 g/L) were added to hot tap water (80 ºC) and vigorously mixed until obtaining a homogenized suspension. After cooling down the mixture to room temperature, the SC was included together with 10% v/v of 'old kombucha' (the medium where the SCOBY was previously maintained). The kombuchas were kept in opened recipients covered with filter paper and stored in darkness at room temperature for 7 days. The resultant beverages (KOM) were kept frozen or freeze-dried until its subsequent use.

2.4. Conventional (cold water, hot water) and advanced (ultrasound-assisted, microwave-assisted) extractions

Two different conventional extraction methods were followed. In both of them, ST powder was mixed in MilliQ water (12 g/L) and vigorously stirred during 15, 30, 45 and 60 min. The processes were carried out at 4 ºC and 95 ºC for cold and hot water extractions, respectively.

Similarly, two different advanced extraction procedures were performed. The solute-solvent ratio was the same (12 g/L), but the extraction times applied were shorter than in conventional methods: 2.5, 5, 7.5, and 10 min. Ultrasound-assisted extractions were developed using an UP 400S (400W, 24 kHz) ultrasonic processor (Hielscher Ultasonics, Teltow, Germany) operating at 60% amplitude. The process was carried out at 4 ºC and a water bath with ice was used to avoid temperature increases. Microwave-assisted extractions were performed in a household microwave oven (Balay 3WM-1926E, Balay, Zaragoza, Spain) operating at 240 W.

All the extractions were carried out in duplicate and the obtained extracts were frozen or freeze-dried and stored at -20 ºC until further analysis.

2.5. Determination of total phenolic compounds content

The total phenolics content (TPC) of KOM and the extracts was measured using the Folin-Ciocalteu method, following the method described in Tejedor-Calvo and Morales (2023) (Tejedor-Calvo & Morales, 2023). Briefly, the samples (50 µL) were mixed with 1.48% HCl (300 µL) and methanol (150 µL) and centrifuged (2 min, 13000 rpm) in a Heraus Pico 21 centrifuge (Thermo Fisher Scientific, Madrid, Spain). Supernatants (50 µL) were added to Na_2CO_3 (2% w/v, 1 mL), vigorously stirred, incubated at room temperature for 3 min and mixed with Folin-Ciocalteu reagent (25 µL). Absorbances were read at 750 nm after a 30 min incubation in a Genesys 10 UV spectrophotometer (Thermo Fisher Scientific, Madrid, Spain). Gallic acid (0.015 to 0.5 mg/mL) was used as standard for quantification (regression equation $y = 2.1478x + 0.0642$). After calculating the TPC in all the extracts, the ones with the highest levels were selected for further analysis, named according to the used extraction procedure: CWE (cold water extract), HWE (hot water extract), UCWE (ultrasound-assisted cold water extract), MHWE (microwave-assisted hot water extract).

2.6. Determination of total carbohydrate content

Total carbohydrate content (TCC) of KOM, CWE, HWE, UCWE and MHWE was determined by the phenol-sulfuric acid method detailed in Smiderle et al. (2017) (Smiderle et al., 2017). The samples were thoroughly diluted in water and were added (25 µL) in triplicate to a 96-well plate, followed by a 5% phenol solution (25 µL) and concentrated H_2SO_4 (125 µL). The plate was sealed and incubated at 80 ºC for 30 min, and absorbance was read at 490 nm using a M200 Plate Reader (Tecan, Mannedorf, Switzerland). D-glucose (0.032 to 0.8 mg/mL) was used as standard for quantification (regression equation $y = 1.2919x + 0.0597$; $R^2 = 0.9997$).

2.7. Determination of soluble proteins content

The total soluble protein content of KOM, CWE, HWE, UCWE and MHWE was determined using the Bradford method reagents (Sigma-Aldrich, Madrid, Spain), according to the manufacturer's instruction manual. In summary, samples were added (10 µL) in triplicate to a 96-well plate, followed by 200 µL of Bradford reagent (1:4 dilution) and incubated for 5 min at RT. Absorbance was measured at 595 nm in a M200 Plate Reader (Tecan, Mannedorf, Switzerland). Bovine serum albumin (0.0125 to 0.5 mg/mL) was used as standard for quantification (regression equation $y = 1.7747x + 0.0185$; $R^2 = 0.9986$).

2.8. HPLC-PAD phenolic compounds analysis

The phenolic compounds characterization of the samples (KOM, CWE, HWE, UCWE and MHWE) was carried out on an Agilent HPLC 1260 Infinity series system equipped with a PAD system (Agilent Technologies Inc., Santa Clara, CA, USA) as reported in Villalva et al. (2018) (Villalva et al., 2018). Briefly, the system was set up with an ACE Excel 3 Super C18 column (150 mm × 4.6 mm, 3 µm particle size) (VWR International, Llinars del Vallés, Spain) protected by a guard column (ACE 3 C18-A, 10 mm × 3 mm) (Symta, Madrid, Spain) at 35 °C. The mobile phase consisted of 0.1 % (v/v) acidic milli-Q water (formic acid) as solvent A and acetonitrile (100%) as solvent B, keeping a constant flow rate of 0.5 mL/min. Prior to injection (20 µl), the freeze-dried samples were dissolved in DMSO and filtered through a 0.45 µm PVDF filter. Finally, quantification of the main compounds was carried out using calibration curves of authentic analytical standards (HPLC purity ≥ 95%), except for catechin derivatives, anthocyanin derivative and the hydroxybenzoic acid derivative, where catechin, oenin chloride, and 3,4-dihydroxybenzoic acid were used, respectively. The standard concentrations were between 0.0002 and 0.5 mg/mL and the following regression equations were obtained and applied: arbutin $y = 32937x – 33.729$ ($R^2 = 0.9997$); catechin $y = 23600 x – 268.91$ ($R^2 = 0.999$); oenin chloride $y = 32937x – 33.729$ ($R^2 = 0.9998$); 3,4-dihydroxybenzoic acid $y = 51853x + 14.631$ ($R^2 = 0.9999$); gallic acid $y = 105746x – 6.0637$ ($R^2 = 0.9998$).

2.9. Antioxidant activities: determination of free radical scavenging activity and cellular antioxidant activity (CAA) testing

The DPPH• scavenging activity of KOM, CWE, HWE, UCWE and MHWE was determined following the procedure described by Morales et al. (2018) (Morales et al., 2018), with slight modifications. The samples were mixed at different concentrations (1.5-100 µL/mL) with a methanolic DPPH• solution (76 nM). Absorbance (517 nm) was read after 15 min of incubation at room temperature in darkness in a M200 Plate Reader (Tecan, Mannedorf, Switzerland). The IC50 was established using the linear correlation obtained with increasing sample concentrations and compared with Trolox to calculate and express the results as Trolox equivalent antioxidant capacity (TEAC) values.

The cellular antioxidant activity (CAA) of the samples (KOM, CWE, HWE, UCWE and MHWE) was tested in human colorectal adenocarcinoma cell line Caco-2 (ATCC, Manassas, VA). For this purpose, Caco-2 cells were cultured in DMEM (Dubelcco's Modified Eagle's Medium) supplemented with fetal bovine serum (FBS) (10%), penicillin (100 U/mL), streptomycin (100 mg/mL), non-essential amino acids (1%) and L-glutamine (2 mM) (Gibco, Paisley, UK) in humidified air containing 5% CO_2 at 37 ºC.

As a preliminary step before testing the CAA, the cytotoxicity of the samples was assessed using the MTT assay (Mosmann, 1983).

CAA assay was performed following the protocol reported by Wolfe and Liu (2007) with slights modifications (Wolfe & Rui, 2007). Briefly, Caco-2 cells were seeded in 96-well plates at a density of 1.5 x 10^5 cells/mL. After 48 h, media was discarded, and the cells were rinsed with PBS (phosphate buffered solution). Then, subtoxic concentrations of the samples and the fluorescent marker DCFH-DA (25 µM) were added to the cells and the plate was incubated for 1 h. After that, media was removed, the cells were washed with PBS. Then, the free radical initiator ABAP (600 µM) was added, and fluorescence was measured every 5 min for 1 h keeping excitation/emission wavelengths of 485/538 nm for a total of 13 cycles (BioTek Cytation 5 plate reader, BioTek Instruments, Winooski, VT, USA). Inhibition percentage (%) was calculated as follows:

$$\% \, inhibition = 1 - \frac{(AUCsample)}{(AUCcontrol)} \, x \, 100 \quad \textbf{(Eq. 1)}$$

IC_{50} value corresponded to a 50% inhibition.

2.10. Macrophage cultures and anti-inflammatory activity testing

The anti-inflammatory activity of KOM, CWE, HWE, UCWE and MHWE was tested in Human THP-1 monocytes (ATCC, Manassas, VA, USA) following the method reported in Villalva et al. (2018). For this purpose, THP-1 were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), streptomycin (100 mg/mL), L-glutamine (2 mM), non-essential amino acids (1%) and penicillin (100 U/mL) (Gibco, Paisley, UK), at 37 ºC in humidified air containing 5% CO_2. TPH-1 cells were seeded in 24 wells plate at a density of $5x10^5$ cells/well with PMA (100 ng/mL) for 48 h, to induce differentiation into macrophages. Prior to the anti-inflammatory activity determination, the toxic effect of the samples was tested on differentiated macrophages employing the MTT assay (Mosmann, 1983).

To perform the immunomodulatory assay, differentiated cells were rinsed with non-supplemented RPMI 1640 medium and incubated with subtoxic concentrations of the extract in the presence of LPS (0.05 μg/mL) for 24 h. After that, the supernatants were recovered and kept frozen at -80 ºC. Then, the secretion of cytokines (TNF-α, IL-1β, IL-6) was measured in the collected supernatants using an ELISA kit (BD Biosciences, Aalst, Belgium), following the manufacturer's instructions.

2.11. Statistical analysis

Differences were evaluated at a 95% confidence level ($P \leq 0.05$) using a one-way analysis of variance (ANOVA) followed by the Tukey multiple comparison test. Statistical analysis was performed using GraphPad Prism version 9.5.1 (GraphPad Software, San Diego, CA, USA). MetaboAnalyst 5.0 software (http://www.metaboanalyst.ca) was used for further data analysis and comparison. Sum normalization and autoscaling were performed. Afterwards, plots of principal component analysis (PCA) or partial least squares discriminant analysis (PLS-DA) were computed to estimate variances, sample distribution, and homologies between sample groups. Pearson correlations were carried out to establish compounds correlation.

3. Results and Discussion

3.1. Conventional and advanced extraction technologies to obtain phenolic-rich fractions

Although many procedures aiming phenolic compound extraction from ST fruits utilized different concentrations of methanol or ethanol as solvents (Morales, 2022), other works demonstrated that cold or boiling water can be efficient as extractant (Masmoudi et al., 2020; Pinheiro et al., 2020). Since some authors demonstrated that a thermal degradation may occur in fruit phenolics when temperature was over 90 ºC (de Lima Marsiglia et al., 2023), both cold and hot water extractions were performed in the current study testing different process times (from 15 to 60 min). When water as 4º C was used (Figure 1a), a clear increase in phenolic concentration of the aqueous extracts was observed during the first 45 min, doubling the amount between 15 and 45 min, from 8.59 to 17.50 mg/100 mL. However, there was not significant differences between 45- and 60-min extractions, so this extract (CWE) was selected for further analysis. A very similar tendency was noticed for extractions using water at 95 ºC (Figure 1b), but the contents were higher than for low temperature procedures: 15.63 mg/100 mL at 15 min and 24.31 mg/100 mL at 45 min, with no significant increase at 60 min. Therefore, the high temperature seemed to not be aggressive enough to degrade phenolic molecules, at least until 45 min, selecting this fraction (HWE) for the following steps. The increase in TPC comparing HWE to CWE can be explained by the release of poorly soluble phenolic compounds at high temperatures, when the lignin bonds to phenolic acids are broken and the lignin itself is degraded giving rise to additional phenolic counts (Antony & Farid, 2022). Masmoudi et al. (2020) subjected ST fruits to boiling water extraction for 30 min and 15 min of incubation at RT, obtaining, after freeze-drying, 23.4

mg GAE/g extract (Masmoudi et al., 2020). These values were in concordance with the obtained in the current work, although slightly lower, since CWE and HWE contained 41.7 and 38.0 mg/g extract, respectively. These differences may be attributed to the different origin of ST fruits and therefore, geographical, climatological, soil, etc., conditions (Morales, 2022). Moreover, as previously mentioned, other authors utilized methanol- and ethanol-mixtures. For instance, Salem et al. (2018) achieved higher levels by using 80% ethanol and 80% methanol, reaching 204.5 and 180.8 mg GAE/g extract, respectively (Salem et al., 2018). However, the alternative extractions that are described in this work avoid the use of these solvents within the framework of greener procedures.

The option that was purposed in this work to increase this extraction ratio was the use of advanced and environmentally friendly methods. In the case of UAE (Figure 1c), carried out at 4 ºC, the extraction time was significantly shortened, since levels up to 20.55 mg phenolics/100 mL were reached with only 7.5 min-processes. The cavitation phaenomena that occur during sonication allowed the disruption of ST cell walls, increasing the extraction rates compared to CWE. However, tested higher times (10 min) did not lead to higher TPCs, suggesting that less soluble phenolics might require higher temperature conditions. Considering this, the extract obtained at 7.5 min was chosen (UCWE). In the case of MAE (Figure 1d), the effect on phenolic extraction enhancement was remarkable. Microwaves acted as heat source accelerating the process and significantly reducing the thermal gradient and the extraction time. As a matter of fact, 5 min were enough to reach 40.73 mg phenolics/100 mL, selecting this fraction (MHWE) for further analysis, since longer times did not produce a significant increase in TPC. These results suggested that MAE was the most efficient and fast technique to obtain phenolic-rich fractions from ST.

These technologies have been scarcely applied to ST fruits, being more frequently used for ST leaves. Nonetheless, El Cadi et al. (2020) used sonication (45 min) to extract defatted ST fruits with ethyl acetate and 80% methanol in a sequential method, recovering fractions with TPC up to 127.5 mg GAE/g. However, these values are hardly comparable to those obtained in the current work, since their procedure was substantially different, including a delipidating process with hexane and the use of organic solvents and longer times for the extraction (El Cadi et al., 2020). Albuquerque et al. (2018) carried out an exhaustive analysis of the optimal conditions to obtain extracts from ST fruits by UAE and MAE. In the case of UAE, the optimal value of TPC was 163.1 mg GAE/g requiring short times, 5 min, similar to those applied in the current experiment, but needing absolute ethanol instead of water to reach these values. For MAE, the optimal level was 164.5 mg/g, achieved with water but with significantly longer time, over 18 min (Albuquerque et al., 2018). Considering these references, the procedures applied in our work demonstrated to be efficient to reach relevant TPCs with short times and absence of organic solvents.

3.2. Characterization of the total carbohydrate, soluble proteins, and phenolic content of ST kombucha and extracts

Regarding the total carbohydrate content (TCC) (Table 1), KOM showed significantly higher values, as expected, since in the elaboration of ST kombucha, 70 mg/mL of cane sugar was added, but also 12 mg/mL of ST (which approximately contained 80% of carbohydrates) and the SC itself, that also provided microbial carbohydrates. Fermentation process reduced TCC from 108.0 mg/mL at the initial stage to 73.1 mg/mL after 7 days. This value was slightly higher than those reported in Tejedor-Calvo and Morales (2023), reaching 58.7 mg/mL after 7 days in a kombucha prepared with the same SC but with ST fruits from a different season, possibly leading to these compositional differences (Boussalah et al., 2018; Tejedor-Calvo & Morales, 2023). In both cases, this content is lower than those registered for other beverages such as fruit juices and carbonated drinks (90-100 mg/mL) (Lin et al., 2018) and even than kombuchas prepared with the same SC and tea or other fruits such as cherries, bottled also after 7 days of fermentation (80-100 mg/mL) (Morales et al., 2023). On the other hand, the water extracts showed significantly lower TCC, since there were no exogenous addition of sugar or microbial carbohydrates. These levels were particularly reduced in CWE (2.2 mg/mL) and, when temperature was higher and/or ultrasounds/microwaves were applied, solubilization and extraction of the carbohydrates were enhanced, registering the maximum value in MHWE (7.9 mg/mL). This fact was expected, since ST fruits contained a relevant amount of dietary fibres and polysaccharides that are not easily extractable and require more drastic conditions (Masmoudi et al., 2020).

Although the objective of the extractions was not linked to the obtention of protein-rich fractions, aqueous MAE was highlighted as an efficient technique recovering soluble proteins (Table 1). Consequently, MHWE showed contents more than 10-times higher than the rest of extracts (106.12 μg/mL), suggesting that the rapid increase in medium temperature was crucial to the release of ST fruits protein structures. UCWE registered the second highest value (9.69 μg/mL), also validating the importance of the disruption of vegetal cell walls to recover intracellular proteins and peptides. Attending to KOM, the protein content was slightly lower (8.66 μg/mL) than in UCWE but higher than in traditional and commercial tea kombuchas and even than in ST fruit kombuchas, being around 3 μg protein/mL in previous studies (Kaashyap et al., 2021; Tejedor-Calvo & Morales, 2023).

The TPC in the water extracts was previously discussed in subsection 3.1., but surprisingly high levels (70.99 mg/100 mL) were registered in KOM when compared to the rest of samples. This concentration is higher than those reported in Tejedor-Calvo & Morales (2023), in a kombucha with the same SC but ST fruits from a different season. In this case, the TPC after 7 days of fermentation was 18.75 mg/100 mL and the reasons explaining this variability might be the same than for TCC, linked to the different seasonality of the collected fruits, that differently affected to their composition (Boussalah et al., 2018). This high recorded TPC surpassed also values reported for tea kombuchas (23.2 mg/100 mL) and fruit kombuchas, for instance, prepared with cherry (16.5 mg/100 mL), strawberry (13.7 mg/100 mL), grape (12.3 mg/100 mL) or plum (9.4 mg/100 mL) (Morales et al., 2023).

Attending to the identification and quantification in terms of specific phenolic compounds (Table 2), a total of 14 different compounds were detected by HPLC-PAD. In this regard, the total amount of phenolic compounds quantified by HPLC-PAD was correlated with the results obtained by the Folin Ciocalteu method. Slightly lower values can be observed when these compounds are quantified by HPLC-PAD in comparison with the Folin Ciocalteu protocol. These differences can be mainly attributed to overestimations in the Folin-Ciocalteu method, as the reagent can react with other molecules such as sugars o aminoacids, among others. Moreover, in a lesser extent, these divergences can be also explained by the impossibility of numerically consider phenolic species under the chromatographic limits of quantification and/or detection. Arbutin (Rt = 10.159 min) was detected in all the samples, although KOM contained only traces, being temperature a key point in its extraction, since the highest values were registered in HWE (1.45 mg/100 mL) and MHWE (1.77 mg/100 mL). These results were in concordance with the effect of used temperature on TPC and previous works that also applied heat to extraction procedures to recover arbutin from ST leaves (Jurica et al., 2017), and other sources such as amaranth (*Amaranthus cruentus*), confirming that higher temperatures led to higher recoveries (Ahmed et al., 2020). This behaviour was also observed for gallic acid (Rt = 11.070 min), appearing in higher concentrations in hot extracts (0.32 and 0.36 mg/100 mL in HWE and MHWE, respectively), although, in this case, KOM showed even higher levels (0.42 mg/100 mL). Gallic acid derivatives were also detected, one of them eluting at 10.461 min and present in higher concentrations in MHWE and KOM (0.26 and 0.25 mg/100 mL, respectively); and the other one eluting at 10.915 min being more concentrated in hot water extracts, reaching 2.93 and 2.55 mg/100 mL in MHWE and HWE, respectively. An hydroxybenzoic acid derivative also eluted (Rt = 11.705 min) in aqueous extracts, being undetectable in KOM. In this case, HWE showed the highest content (0.17 mg/100 mL), suggesting that microwave treatment might moderately affect its stability. However, the most noticeable differences were registered in three different catechin derivatives (Rt = 12.232 min, 13.531 min and 15.810 min), that are mainly concentrated in KOM (20.03, 19.99 and 20.28 mg/100 mL) in comparison to the aqueous extracts, that showed ranges between 1.65 and 4.23 mg/100 mL with higher amounts in MHWE and in HWE than in cold extracts. Considering the results, these catechin derivatives seemed to be better extracted with temperature but it can be also deduced that the kombucha elaboration and fermentation directly impact in the concentration of these phenolic species. First, the fermentative action of the SC microorganisms has demonstrated to be effective releasing phenolics to the media and increasing TPC during the first week of fermentation, as reported for ST fruits kombucha (Tejedor-Calvo & Morales, 2023) but also kombuchas prepared with tea and other fruits (Ayed et al., 2017; Kaewkod et al., 2019; Morales et al., 2023). The SC activity, together with low pH, can contribute to enzymatic release of phenolic species through acid hydrolysis, depolymerization, etc. and also to the degradation of vegetal cell wall components (lignin, cellulose, hemicellulose, etc.) augmenting TPC counts (Szymanska-Chargot et al., 2017). Moreover, since the greater differences were associated with catechin derivatives, the 'old kombucha' which is added during KOM preparation might be responsible for the higher concentration of these molecules. 'Old kombucha' is the media where the SC is maintained before its use in kombucha recipes, and it results from previous fermentations normally carried out using tea as substrate. Consequently, the relevant content of catechins and catechin derivatives in tea leaves (Meyer et al., 2023) might explain their presence and amount also in alternative kombuchas such as KOM.

3.3. Antioxidant activity of ST kombucha and extracts

The antioxidant capacity of fractions obtained from ST fruits has been widely demonstrated through *in vitro* assays that in most cases involved radical scavenging activity experiments or studied the oxidation of food components (lipids, proteins, etc.) when added to food products (burgers, sausages, etc.), noticing high correlations between this antioxidant activity and their phenolic content (Armenteros et al., 2013; El Cadi et al., 2020; Ganhão et al., 2010, 2013).

KOM and aqueous extracts were evaluated, at first, in terms of their $DPPH^{\bullet}$ scavenging activity, recording TEAC values ranging from 1.12 to 2.76 µmol Trolox/mL (Table 3), being the highest for KOM (2.63 µmol/mL) and MHWE (2.76 µmol/mL), which were the samples with higher TPC, gallic acid and catechin derivatives content. In the case of MHWE, the calculated IC_{50} (6.32 µL/mL) was significantly lower than in the rest of extracts. Expressed as µg extract/mL to allow comparisons with previous works that carried out $DPPH^{\bullet}$ scavenging assays in ST fruits extracts, it was also much lower (61.3 µg/mL) than those reported, for instance, by Salem et al.

(2018), who obtained ethanolic and methanolic extracts with IC_{50} values of 324.1 and 379.5 µg/mL (Salem et al., 2018). Very similar values to those registered for MHWE were obtained in a phenolic-rich fraction extracted with 80% methanol (60.9 µg/mL) (Guimarães et al., 2014). This solvent was also used by El Cadi et al. (2020) that previously defatted the fruits with hexane, to obtain significantly lower IC_{50} values (1.4 µg/mL) (El Cadi et al., 2020). However, as mentioned for the phenolic extraction, the procedures described in the current work, particularly MAE, demonstrated to be effective recovering radical scavenging fractions using short times and water as GRAS and environmentally friendly solvent. Regarding KOM, this TEAC value was interesting and promising, being slightly lower than tea kombucha (3.59 µmol Trolox/mL) prepared with the same SC, but higher than fruit kombuchas obtained following the same procedure, for instances, with strawberry (2.05 µmol/mL at day 2, but 0.49 µmol/mL at day 7) or cherry (0.41 µmol/mL) (Morales et al., 2023).

Nonetheless, although radical scavenging assays provide interesting results as preliminary studies, it does not simulate the biological environment, since antioxidant molecules must reach their targets passing though cell membranes (Tejedor-Calvo et al., 2022). For this reason, CAA assay was performed in Caco-2 cells, leading to significantly higher antioxidant capacity in KOM (Table 3), reaching CAA values of 6.22 µmol quercetin equivalents/mL, being 10- to 22-times higher than those registered for aqueous extracts. This fact may reinforce the importance of the catechin derivatives identified in KOM as potent antioxidants that can pass through Caco-2 cell membranes exerting their activity. Regarding the IC_{50}, MHWE registered the second most potent activity (66.0 µL/mL), being correlated with radical scavenging and TPC results. When the IC_{50} units were transformed into µg extract/mL, the range was between 605.9 and 672.9 µg/mL. No publications could be found reporting similar values for CAA in ST fruits or extracts, but the analysed samples showed much higher antioxidant power when compared, for instance, with the CAA of vegetables such as tomatoes, carrots, or potatoes, among others (Song et al., 2010). When the same assay was carried out to evaluate subcritical water extracts obtained from truffles (*Tuber aestivum* and *Terfezia claveryi*), slightly lower IC_{50} values were reported

(402.96 and 565.95 μg/mL, respectively), indicating that advanced extractions using water as solvent can be applied to other sources to obtain antioxidant fractions (Tejedor-Calvo et al., 2022).

Despite the lack of published results of ST extracts CAA, two antioxidant activity assays were carried in human cell models: Mendes et al. (2011) incubated human erythrocytes with water extracts obtained from ST fruits and leaves, exerting high antioxidant capacities by inhibiting lipid peroxidation and oxidative haemolysis (Mendes et al., 2011); Jurica et al. (2018) obtained an extract from ST leaves by aqueous UAE that inhibited lipid peroxidation in human peripheral blood lymphocytes (Jurica et al., 2018).

Moreover, to investigate deeper correlations, a heatmap was built comparing the IC_{50} values in DPPH and CAA IC_{50} versus the TPC detected by HPLC and Folin-Ciocalteu method and also the individual compounds identified by HPLC (Figure 2).

The DPPH IC_{50} showed interesting negative correlations, besides the expected ones with TPC calculated by the colorimetric protocol (r = -0.888, P < 0.0001) as well as the chromatographic method (r = -0.600, P < 0.05), validating the ability of these compounds to scavenge free radicals such as DPPH$^{•}$. Some specific molecules showed also negative correlations, such as catechin derivatives 2 (r = -0.676, P < 0.01), 3 (r = -0.664, P < 0.01) and 4 (r = -0.650, P < 0.01). The CAA IC_{50} showed even stronger negative correlations with TPC calculated by Folin-Ciocalteu method (r = -0.878, P < 0.001) and particularly HPLC (r = -0.968, P < 0.0001), as expected, confirming the relevance of the high phenolic concentrations in antioxidant activity in cellular models. Attending to specific compounds, the most negative correlations were observed for the same catechin derivatives that were mentioned for DPPH$^{•}$: derivative 2 (r = -0.950, P < 0.0001), 3 (r = -0.946, P < 0.0001) and 4 (r = -0.940, P < 0.0001), being these molecules mainly present in KOM, but also in the extracts, particularly those obtained using high temperatures, HWE and MHWE. Among the additional molecules that exerted strongly negative correlations with CAA IC_{50}, gallic acid showed the most remarkable values (r = -0.810, P < 0.001). Therefore, together with the capacity to scavenge DPPH$^{•}$, these molecules were able to reach their targets inside the cells, placing kombucha elaboration and hot extractions as the most adequate techniques to obtain antioxidant fractions from ST fruits.

3.4. Anti-inflammatory activity of ST kombucha and extracts

Attending to the ability of inhibiting the secretion of proinflammatory cytokines exerted by KOM and the aqueous extracts (Figure 3), interesting results were observed: TNF-α levels were reduced by both KOM and the extracts, that were more efficient than KOM (52-66% reduction vs. 31% reduction); IL-1β secretion was not decreased by KOM, but all the extracts were capable of obtaining 21-24% reductions, with no significant differences between the extracts; IL-6 production was inhibited by both KOM and extracts (70-88% decreases) with no statistical differences between samples.

These results suggest that, although KOM showed higher CAA, the extracts were more versatile impairing pro-inflammatory cytokines production by THP-1 macrophages. Therefore, the levels of specific immune-modulatory molecules (phenolic compounds but also other metabolites) might be crucial to explain the observed variations. Just a few studies can be found addressing anti-inflammatory properties of ST extracts, and most of them were focussed on ST leaves. In this sense, a phenolic-rich fraction obtained extracting ST leaves with methanol exerted inhibitory activity against the activation of STAT1 protein mediated by IFN-γ in human breast cancer cells, fibroblasts and alveolar epithelial cells. STAT1 is a complex molecule that might provoke exacerbated inflammatory responses due to its excessive or wrong activation. Moreover, this extract inhibited STAT3 activation elicited by IL-6 in THP-1 cells, being this action considered beneficial to ameliorate inflammatory processes. These *in vitro* results encouraged *in vivo* tests in CD mice with carrageenan-induced pleural and lung inflammation. When pleural exudates were collected from the group orally treated with the extract, levels of TNF-α, IL-1β and IL-6 were decreased (Mariotto, Ciampa, et al., 2008; Mariotto, Esposito, et al., 2008), in concordance with the obtained results in this work for ST fruits extracts tested in THP-1 cells.

Regarding KOM, this was the first time that ST kombucha anti-inflammatory activity was assessed. Kombuchas prepared with other substrates, such as black tea or oak leaves, also showed interesting immune-modulatory properties. Villarreal-Soto et al. (2019, 2020) demonstrated that black tea kombucha inhibited 5-lipooxigenase activity, an enzyme related with fatty acids conversion into leukotrienes (Villarreal-Soto et al., 2019, 2020) and Vázquez-Cabral et al. (2017) reported a reduction in TNF-α and IL-6 secretion and nitric oxide production when incubated hydrogen peroxide-activated THP-1 macrophages with oak leaves kombucha (Vázquez-Cabral et al., 2017).

When correlation analyses among anti-inflammatory and antioxidant activities together phenolic compounds content were carried out, more difficulties were found to extract significant results, since many of the values were artefacts with no biological sense (Figure 4). Besides the high positive correlations observed between cytokines, arbutin showed negative correlation with TNF-α secretion (r = -0.41), but non-significant (P = 0.13). Therefore, the absence of significant negative correlations between TPC or individual phenolic compounds and cytokines secretion or even positive correlations between IC_{50} (in $DPPH^{\bullet}$ and CAA assays) and cytokines levels, suggested that other bioactive compounds, probably essential oils or fatty acids, from ST fruits might be acting as immune-modulatory agents. Since previous research has been mainly focused on ST phenolics bioactivities, further investigation targeting other molecules such as peptides, lipids, carbohydrates must be encouraged.

4. Conclusions

Green extraction technologies using water as solvent, as well as kombucha fermentation, demonstrated to be effective to obtain phenolic-rich fractions from ST fruits with antioxidant and anti-inflammatory activities.

First, the use of high temperatures was useful to increase phenolic extraction without noticing degradation events, at least in most of tested times. When advanced methods were applied, as those using ultrasounds and microwaves, the extraction rates were increased, and the required times were shortened. MAE must be particularly highlighted, leading to higher TPC but also higher total carbohydrates and soluble proteins contents.

Moreover, kombucha beverages prepared with ST fruits showed also very interesting composition, suggesting that microbial action and low pH during fermentation time (7 days) contributed to release higher amounts of TPC and specific molecules such as catechin derivatives 2, 3 and 4.

Regarding biological activities, antioxidant capacity of KOM and aqueous extracts was validated as radical scavengers but also as antioxidant agents that were able to pass through cellular membranes and reach their targets. In this sense, the high antioxidant activity of KOM must be mentioned, followed by MHWE, observing also strong negative correlations between TPC or individual species (catechin derivatives 2, 3, 4, gallic acid) and antioxidant IC_{50} values. These correlations were not significant applied to anti-inflammatory activities, but the extracts reduced all tested cytokines secretion and KOM was effective decreasing TNF-α and IL-6 levels, suggesting that additional bioactive molecules together with phenolic compounds might be crucial for immune-modulatory effects.

Considering all these results, the main hypothesis of the study was validated, confirming that the novel obtained ST fractions exerted more potent effects than conventional extracts. However, further investigation should be encouraged through *in vivo* experiments to corroborate the promising activities exerted in cellular models, but also to identify the specific molecules responsible for anti-inflammatory capacity.

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Author contributions

María de las Nieves Siles-Sánchez: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft. **Eva Tejedor-Calvo:** Formal analysis, Investigation, Writing – original draft, Writing – review & editing. **Laura Jaime:** Conceptualization, Methodology, Funding acquisition, Supervision, Writing – review & editing. **Susana Santoyo:** Conceptualization, Methodology, Funding acquisition, Supervision, Writing – review & editing. **Diego Morales:** Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing.

Statements and declarations

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Data availability statement

Research data are not shared.

Conflict of interest statement

The authors declare no financial, non-financial, competing interests or others that might be perceived to influence the results and/or discussion reported in this paper.

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Tables

Table 1. Composition of strawberry tree kombucha and extracts in terms of total carbohydrate (TCC), soluble proteins and total phenolic compounds (TPC) content. $^{a\text{-}d}$Different letters denote significant statistical differences ($P < 0.05$) for the same compounds between different samples.

Sample	TCC (mg/mL)	Soluble proteins (µg/mL)	TPC (mg/100 mL)
KOM	73.11 ± 1.23^{a}	8.66 ± 0.08^{c}	70.99 ± 2.99^{a}
CWE	2.21 ± 0.30^{d}	3.52 ± 0.24^{e}	17.50 ± 1.00^{d}
HWE	3.89 ± 0.03^{c}	5.98 ± 0.81^{d}	24.31 ± 1.33^{c}
UCWE	4.02 ± 0.21^{c}	9.69 ± 0.48^{b}	20.55 ± 0.66^{cd}
MHWE	7.89 ± 0.56^{b}	106.12 ± 0.20^{a}	40.72 ± 1.33^{b}

Table 2. Phenolic composition (mg/100 mL sample) of strawberry tree kombucha and extracts. $^{a-e}$Different letters denote significant statistical differences (P < 0.05) for the same compound between different samples. Rt = retention time

	Rt	Compound	Average ± SD				
			KOM	CWE	HWE	UCWE	MHWE
1	10.159	Arbutin*	Traces^{e}	0.91 ± 0.02^{d}	1.45 ± 0.01^{b}	1.27 ± 0.01^{c}	1.77 ± 0.01^{a}
2	10.461	Gallic acid derivative (1)	0.25 ± 0.00^{a}	0.13 ± 0.01^{c}	0.20 ± 0.00^{b}	0.19 ± 0.00^{b}	0.26 ± 0.00^{a}
3	10.915	Gallic acid derivative (2)	1.76 ± 0.00^{d}	1.57 ± 0.00^{e}	2.55 ± 0.00^{b}	1.99 ± 0.00^{c}	2.93 ± 0.01^{a}
4	11.070	Gallic acid*	0.42 ± 0.00^{a}	0.21 ± 0.00^{b}	0.32 ± 0.00^{c}	0.23 ± 0.00^{d}	0.36 ± 0.00^{b}
5	11.705	Hydroxybenzoic acid derivative	n.d.^{e}	0.04 ± 0.00^{d}	0.17 ± 0.00^{a}	0.06 ± 0.00^{c}	0.09 ± 0.00^{b}
6	11.828	Catechin derivative (1)	n.d.^{e}	1.04 ± 0.00^{d}	1.60 ± 0.00^{c}	1.66 ± 0.01^{b}	2.53 ± 0.00^{a}
7	12.232	Catechin derivative (2)	20.03 ± 0.01^{a}	2.04 ± 0.00^{e}	2.81 ± 0.02^{c}	2.54 ± 0.00^{d}	3.75 ± 0.02^{b}
8	12.716	Anthocyanin derivative	n.d.^{d}	0.24 ± 0.00^{b}	0.23 ± 0.01^{bc}	0.20 ± 0.00^{c}	0.56 ± 0.01^{a}
9	13.531	Catechin derivative (3)	19.99 ± 0.01^{a}	1.65 ± 0.00^{e}	2.88 ± 0.01^{c}	2.23 ± 0.04^{d}	3.75 ± 0.01^{b}
10	15.810	Catechin derivative (4)	20.28 ± 0.02^{a}	1.83 ± 0.01^{e}	3.49 ± 0.00^{c}	2.29 ± 0.01^{d}	4.23 ± 0.03^{b}
11	16.335	Catechin derivative (5)	n.d.^{e}	1.09 ± 0.00^{d}	1.75 ± 0.00^{b}	1.72 ± 0.00^{c}	2.50 ± 0.00^{a}
12	16.473	Catechin derivative (6)	n.d.^{e}	1.11 ± 0.00^{d}	1.82 ± 0.00^{b}	1.69 ± 0.00^{c}	2.63 ± 0.00^{a}
13	17.446	Catechin derivative (7)	n.d.^{e}	1.15 ± 0.02^{d}	1.97 ± 0.00^{b}	1.68 ± 0.00^{c}	2.73 ± 0.01^{a}
14	20.298	Catechin derivative (8)	n.d.^{e}	1.08 ± 0.02^{d}	1.64 ± 0.03^{c}	1.73 ± 0.00^{b}	2.49 ± 0.00^{a}
Total			62.70 ± 0.02^{a}	14.10 ± 0.01^{e}	22.80 ± 0.01^{c}	19.49 ± 0.03^{d}	30.59 ± 0.09^{b}

*A standard was used to corroborate the compound identification and quantification.

Table 3. *In vitro* antioxidant activity calculated by DPPH$^{\bullet}$ radical scavenging activity assay ([1]Values expressed as IC_{50} and TEAC) and cellular antioxidant activity (CAA) assay ([2]Values expressed as IC_{50} and CAA) for strawberry tree kombucha and extracts. $^{a\text{-}d}$Different letters denote significant statistical differences ($P < 0.05$) for the same compound between different samples.

Sample	**IC_{50} (µL/mL)[1]**	**TEAC (µmol Tolox/mL)[1]**	**IC_{50} (µL/mL)[2]**	**CAA (µmol QE/mL)[2]**
KOM	6.97 ± 1.96^{b}	2.63 ± 0.67^{ab}	6.76 ± 0.46^{d}	6.22 ± 0.42^{a}
CWE	15.63 ± 1.67^{a}	1.12 ± 0.12^{b}	160.21 ± 13.50^{a}	0.28 ± 0.03^{b}
HWE	11.83 ± 0.48^{a}	1.47 ± 0.06^{b}	102.94 ± 11.07^{b}	0.40 ± 0.03^{b}
UCWE	14.99 ± 0.19^{a}	1.16 ± 0.01^{b}	89.10 ± 4.50^{bc}	0.47 ± 0.02^{b}
MHWE	6.32 ± 0.29^{b}	2.76 ± 0.12^{a}	66.01 ± 7.53^{c}	0.63 ± 0.07^{b}

Figures

Figure 1. Total phenolic content (mg/100 mL) in the fractions obtained by cold water (a), hot water (b), ultrasound-assisted (c) and microwave-assisted (d) extractions at different times (15, 30, 45 and 60 min for cold and hot water extractions; 2.5, 5, 7.5 and 10 min for ultrasound- and microwave-assisted extractions). [a-c]Different letters denote significant statistical differences (P < 0.05) between different extraction times.

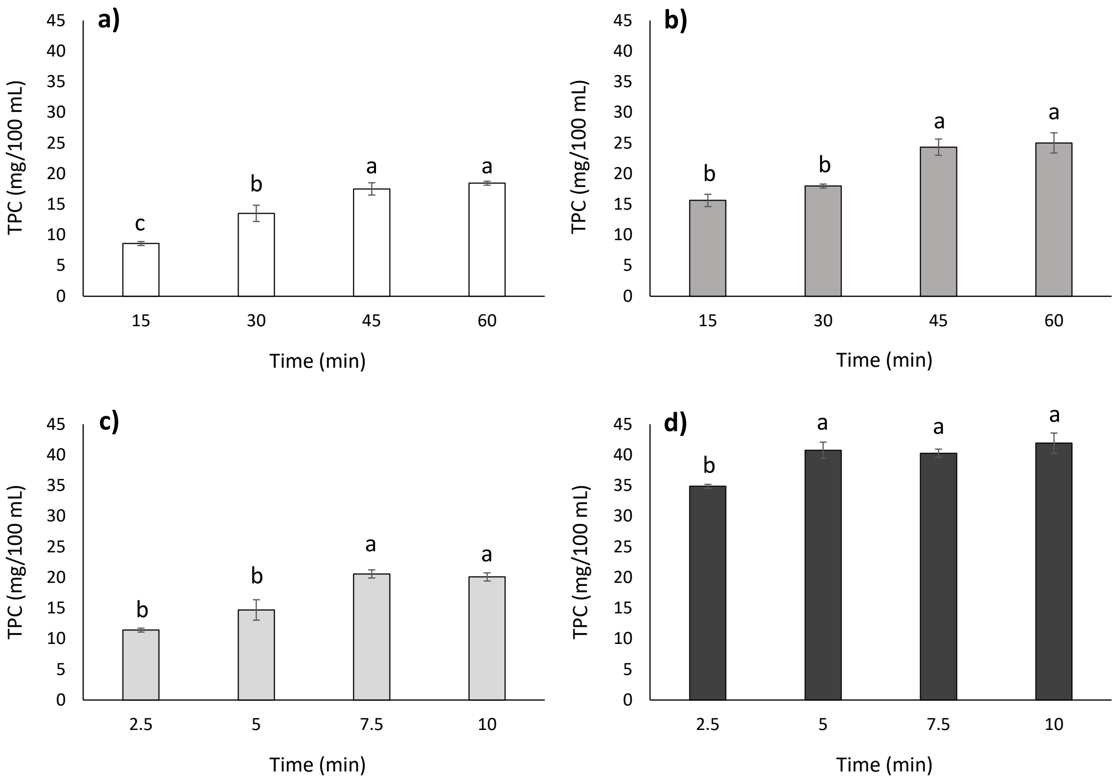


Figure 2. Heatmap of the antioxidant activity (DPPH IC_{50} and CAA IC_{50}) and phenolic compounds. TPC-Folin correspond total phenolic compounds detected by Folin-Ciocalteu method, while TPC-HPLC correspond to the sum of the phenolic compounds detected by HPLC (arbutin, gallic acid, gallic acid derivatives, catechin derivatives, hydroxybenzoic acid derivative, and anthocyanin derivative). Pearson correlation coefficients and p-values are reported in Table S1 and S2 in the supplementary material. TPC = Total phenolic compounds; CAT = Catechin; GA = Gallic acid; HA = Hydroxybenzoic acid; AN = Anthocyanin; der = derivative

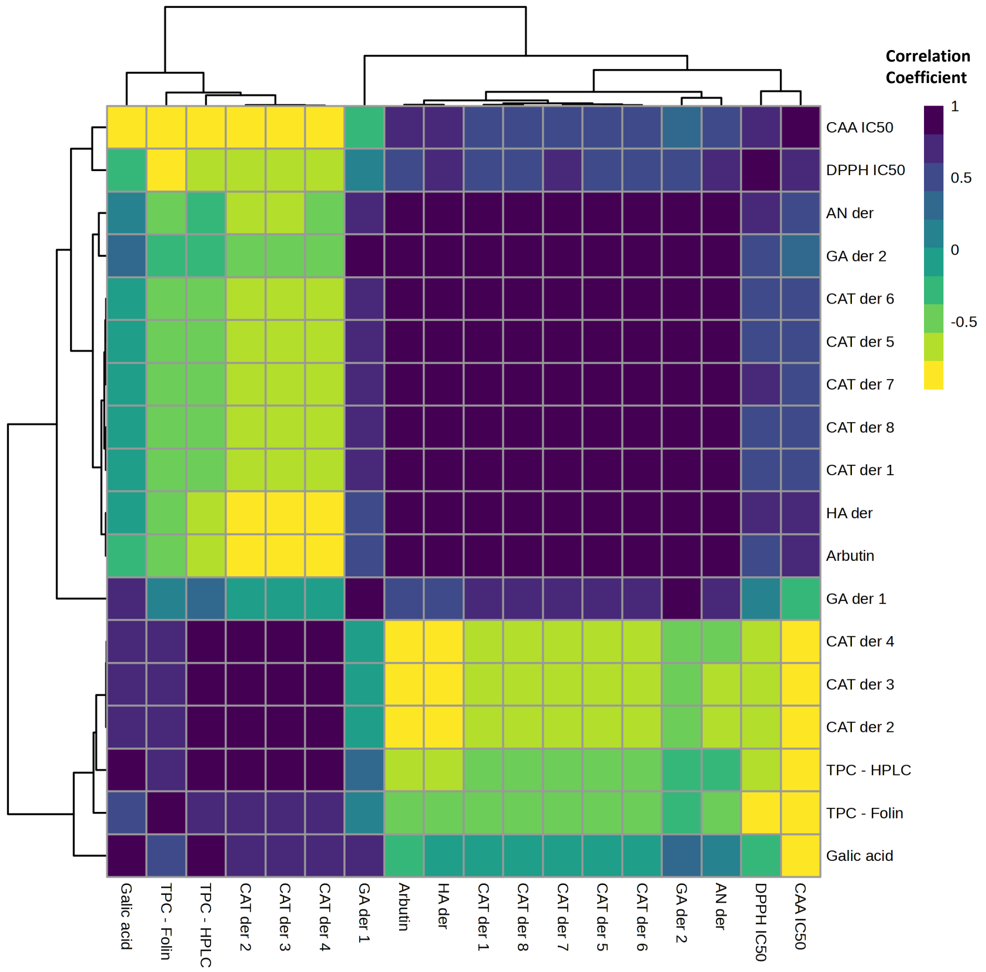


Figure 3. Levels of a) TNF-α, b) IL-1β and c) IL-6 secreted by THP-1 macrophages activated with LPS in presence of strawberry tree kombucha and extracts (30 μg/mL). Positive control (C+): cells stimulated with LPS but in absence of kombucha or extract. Negative control (C-): non LPS-activated cells. Different letters (a-c) denote significant differences ($P < 0.05$) between samples.

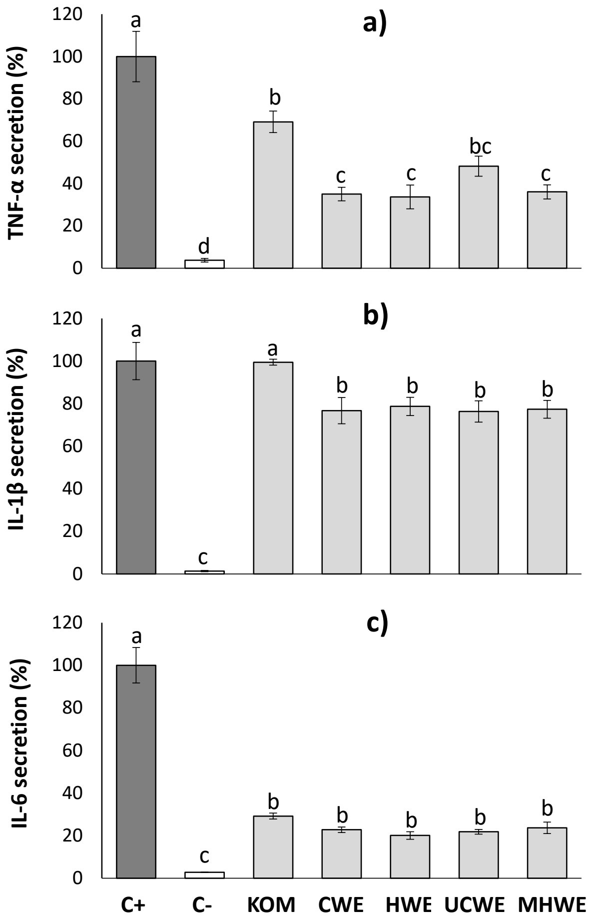
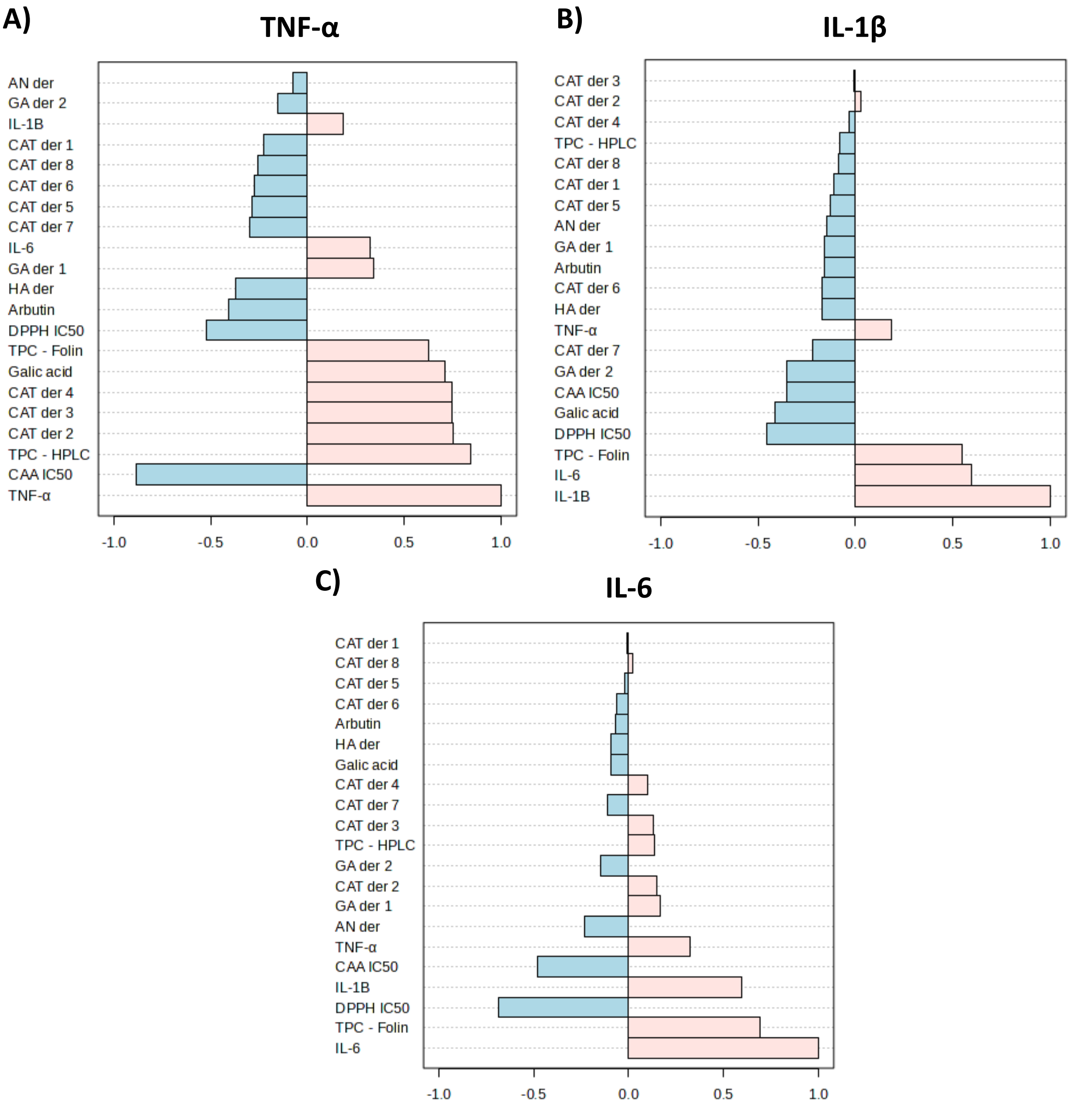


Figure 4. Pearson correlation coefficients of compounds correlated with the secretion of A) TNF-α, B) IL-β and C) IL-6. TPC = Total phenolic compounds; CAT = Catechin; GA = Gallic acid; HA = Hydroxybenzoic acid; AN = Anthocyanin; der = derivative



Supplementary material

Table S1. Correlation table corresponding to the heatmap (Figure 3), including all Pearson correlation coefficients. TPC = Total phenolic compounds; CAT = Catechin; GA = Gallic acid; HA = Hydroxybenzoic acid; AN = Anthocyanin; der = derivative

	Gallic acid	**TPC - Folin**	**TPC - HPLC**	**CAT der 2**	**CAT der 3**	**CAT der 4**	**GA der 1**	**Arbutin**	**HA der**	**CAT der 1**	**CAT der 8**	**CAT der 7**	**CAT der 5**	**CAT der 6**	**GA der 2**	**AN der**	**DPPH IC50**	**CAA IC50**
Gallic acid	1	0.51592	0.87919	0.71579	0.72363	0.72909	0.66276	-0.19772	-0.16114	-0.02468	-0.0621	-0.07294	-0.08063	-0.06094	0.23427	0.09222	-0.31683	-0.81015
TPC - Folin	0.51592	1	0.73222	0.76404	0.74726	0.73082	0.15445	-0.56127	-0.57361	-0.47137	-0.46065	-0.5661	-0.49524	-0.52403	-0.37198	-0.57387	-0.88815	-0.87769
TPC - HPLC	0.87919	0.73222	1	0.9613	0.96412	0.96528	0.26616	-0.63106	-0.60627	-0.48829	-0.51604	-0.5351	-0.53461	-0.52239	-0.2492	-0.37268	-0.60027	-0.96812
CAT der 2	0.71579	0.76404	0.9613	1	0.99947	0.99836	0.00025	-0.81828	-0.80083	-0.70718	-0.7278	-0.747	-0.74421	-0.73619	-0.50619	-0.58563	-0.67574	-0.95021
CAT der 3	0.72363	0.74726	0.96412	0.99947	1	0.99967	0.00489	-0.81381	-0.79561	-0.70227	-0.72398	-0.73937	-0.73937	-0.72992	-0.4964	-0.57839	-0.66417	-0.94567
CAT der 4	0.72909	0.73082	0.96528	0.99836	0.99967	1	0.00653	-0.81088	-0.7917	-0.6989	-0.72172	-0.73365	-0.73627	-0.72543	-0.48967	-0.56936	-0.64985	-0.94036
GA der 1	0.66276	0.15445	0.26616	0.00025	0.00489	0.00653	1	0.57251	0.59117	0.70206	0.68352	0.6454	0.66472	0.66735	0.8401	0.63478	0.03964	-0.25246
Arbutin	-0.19772	-0.56127	-0.63106	-0.81828	-0.81381	-0.81088	0.57251	1	0.99788	0.98372	0.98854	0.98798	0.99261	0.98956	0.90443	0.85081	0.59239	0.64028
HA der	-0.16114	-0.57361	-0.60627	-0.80083	-0.79561	-0.7917	0.59117	0.99788	1	0.98909	0.99056	0.99458	0.99474	0.99471	0.91931	0.88159	0.6218	0.62621
CAT der 1	-0.02468	-0.47137	-0.48829	-0.70718	-0.70227	-0.6989	0.70206	0.98372	0.98909	1	0.99831	0.99316	0.99796	0.99787	0.96093	0.8941	0.55588	0.50487
CAT der 8	-0.0621	-0.46065	-0.51604	-0.7278	-0.72398	-0.72172	0.68352	0.98854	0.99056	0.99831	1	0.98971	0.99904	0.99593	0.948	0.87159	0.535	0.52103
CAT der 7	-0.07294	-0.5661	-0.5351	-0.747	-0.73937	-0.73365	0.6454	0.98798	0.99458	0.99316	0.98971	1	0.99427	0.99848	0.95098	0.90228	0.62312	0.57359
CAT der 5	-0.08063	-0.49524	-0.53461	-0.74421	-0.73937	-0.73627	0.66472	0.99261	0.99474	0.99796	0.99904	0.99427	1	0.99833	0.94639	0.87477	0.55764	0.54731
CAT der 6	-0.06094	-0.52403	-0.52239	-0.73619	-0.72992	-0.72543	0.66735	0.98956	0.99471	0.99787	0.99593	0.99848	0.99833	1	0.95455	0.8948	0.5896	0.54896
GA der 2	0.23427	-0.37198	-0.2492	-0.50619	-0.4964	-0.48967	0.8401	0.90443	0.91931	0.96093	0.948	0.95098	0.94639	0.95455	1	0.88505	0.47433	0.30215
AN der	0.09222	-0.57387	-0.37268	-0.58563	-0.57839	-0.56936	0.63478	0.85081	0.88159	0.8941	0.87159	0.90228	0.87477	0.8948	0.88505	1	0.76398	0.45264
DPPH IC50	-0.31683	-0.88815	-0.60027	-0.67574	-0.66417	-0.64985	0.03964	0.59239	0.6218	0.55588	0.535	0.62312	0.55764	0.5896	0.47433	0.76398	1	0.72807
CAA IC50	-0.81015	-0.87769	-0.96812	-0.95021	-0.94567	-0.94036	-0.25246	0.64028	0.62621	0.50487	0.52103	0.57359	0.54731	0.54896	0.30215	0.45264	0.72807	1

Table S2. P-values corresponding to the heatmap (Figure 3). TPC = Total phenolic compounds; CAT = Catechin; GA = Gallic acid; HA = Hydroxybenzoic acid; AN = Anthocyanin; der = derivative

	Gallic acid	TPC - Folin	TPC - HPLC	CAT der 2	CAT der 3	CAT der 4	GA der 1	Arbutin	HA der	CAT der 1	CAT der 8	CAT der 7	CAT der 5	CAT der 6	GA der 2	AN der	DPPH IC50	CAA IC50
Gallic acid	NA	0.048994	1.58E-05	0.0026915	0.0022915	0.0020423	0.0070875	0.47998	0.56615	0.93044	0.82599	0.79614	0.77515	0.82918	0.40069	0.74376	0.24991	0.00025043
TPC - Folin	0.048994	NA	0.00191	0.00091284	0.0013651	0.0019681	0.58259	0.02948	0.02537	0.076111	0.08398	0.027814	0.060509	0.044953	0.17217	0.025288	9.80E-06	1.71E-05
TPC - HPLC	1.58E-05	0.00191	NA	1.18E-08	7.29E-09	5.90E-09	0.33763	0.011644	0.016578	0.064786	0.048931	0.039837	0.040054	0.045747	0.37042	0.17129	0.017984	3.41E-09
CAT der 2	0.0026915	0.00091284	1.18E-08	NA	0	0	0.9993	0.00019239	0.00033387	0.0031929	0.0020992	0.0013733	0.0014637	0.0017515	0.054188	0.021801	0.0056904	5.92E-08
CAT der 3	0.0022915	0.0013651	7.29E-09	0	NA	0	0.98621	0.00022277	0.00038973	0.0035108	0.0022752	0.0016323	0.0016325	0.0020068	0.059813	0.0239	0.0069242	1.03E-07
CAT der 4	0.0020423	0.0019681	5.90E-09	0	0	NA	0.98156	0.0002447	0.00043639	0.0037434	0.0023844	0.0018516	0.0017486	0.0022071	0.06392	0.026734	0.0087316	1.87E-07
GA der 1	0.0070875	0.58259	0.33763	0.9993	0.98621	0.98156	NA	0.025718	0.02029	0.0035251	0.004963	0.009363	0.0068609	0.0065656	8.86E-05	0.011015	0.88847	0.36399
Arbutin	0.47998	0.02948	0.011644	0.00019239	0.00022277	0.0002447	0.025718	NA	0	4.49E-11	4.65E-12	6.32E-12	2.71E-13	2.54E-12	3.67E-06	5.80E-05	0.019969	0.010133
HA der	0.56615	0.02537	0.016578	0.00033387	0.00038973	0.00043639	0.02029	0	NA	3.37E-12	1.33E-12	3.64E-14	2.98E-14	3.09E-14	1.27E-06	1.40E-05	0.01333	0.012505
CAT der 1	0.93044	0.076111	0.064786	0.0031929	0.0035108	0.0037434	0.0035251	4.49E-11	3.37E-12	NA	0	1.64E-13	0	0	1.26E-08	6.97E-06	0.031423	0.054922
CAT der 8	0.82599	0.08398	0.048931	0.0020992	0.0022752	0.0023844	0.004963	4.65E-12	1.33E-12	0	NA	2.32E-12	0	5.77E-15	7.82E-08	2.31E-05	0.039884	0.046415
CAT der 7	0.79614	0.027814	0.039837	0.0013733	0.0016323	0.0018516	0.009363	6.32E-12	3.64E-14	1.64E-13	2.32E-12	NA	5.17E-14	0	5.37E-08	4.22E-06	0.01308	0.025376
CAT der 5	0.77515	0.060509	0.040054	0.0014637	0.0016325	0.0017486	0.0068609	2.71E-13	2.98E-14	0	0	5.17E-14	NA	0	9.49E-08	1.97E-05	0.03078	0.034715
CAT der 6	0.82918	0.044953	0.045747	0.0017515	0.0020068	0.0022071	0.0065656	2.54E-12	3.09E-14	0	5.77E-15	0	0	NA	3.31E-08	6.69E-06	0.02071	0.03406
GA der 2	0.40069	0.17217	0.37042	0.054188	0.059813	0.06392	8.86E-05	3.67E-06	1.27E-06	1.26E-08	7.82E-08	5.37E-08	9.49E-08	3.31E-08	NA	1.16E-05	0.074037	0.27372
AN der	0.74376	0.025288	0.17129	0.021801	0.0239	0.026734	0.011015	5.80E-05	1.40E-05	6.97E-06	2.31E-05	4.22E-06	1.97E-05	6.69E-06	1.16E-05	NA	0.00091417	0.090231
DPPH IC50	0.24991	9.80E-06	0.017984	0.0056904	0.0069242	0.0087316	0.88847	0.019969	0.01333	0.031423	0.039884	0.01308	0.03078	0.02071	0.074037	0.00091417	NA	0.0020871
CAA IC50	0.00025043	1.71E-05	3.41E-09	5.92E-08	1.03E-07	1.87E-07	0.36399	0.010133	0.012505	0.054922	0.046415	0.025376	0.034715	0.03406	0.27372	0.090231	0.0020871	NA

Table S3. Statistical data of correlation analyses with TNF-α secretion. TPC = Total phenolic compounds; CAT = Catechin; GA = Gallic acid; HA = Hydroxybenzoic acid; AN = Anthocyanin; der = derivative

	Correlation coefficient	P-value
TNF-α	1	0
CAA IC50	-0.88433	1.21E-05
TPC - HPLC	0.84381	7.68E-05
CAT der 2	0.74944	0.0012977
CAT der 3	0.74832	0.001332
CAT der 4	0.74472	0.0014468
Gallic acid	0.70859	0.0031063
TPC - Folin	0.62806	0.012171
DPPH IC50	-0.5235	0.045205
Arbutin	-0.40591	0.13331
HA der	-0.37259	0.1714
GA der 1	0.34267	0.21119
IL-6	0.32242	0.24119
CAT der 7	-0.29784	0.28097
CAT der 5	-0.28636	0.30079
CAT der 6	-0.27472	0.32173
CAT der 8	-0.25741	0.35435
CAT der 1	-0.2242	0.4218
IL-1β	0.1826	0.51481
GA der 2	-0.1537	0.58444
AN der	-0.076195	0.78724

Table S4. Statistical data of correlation analyses with IL-1β secretion. TPC = Total phenolic compounds; CAT = Catechin; GA = Gallic acid; HA = Hydroxybenzoic acid; AN = Anthocyanin; der = derivative

	Correlation coefficient	**P-value**
IL-1β	1	3.88E-103
IL-6	0.5964	0.018938
TPC - Folin	0.54406	0.036028
DPPH IC50	-0.45776	0.086199
Gallic acid	-0.41606	0.12295
CAA IC50	-0.35201	0.1982
GA der 2	-0.35145	0.19895
CAT der 7	-0.21753	0.4361
TNF-α	0.1826	0.51481
HA der	-0.1735	0.53631
CAT der 6	-0.16968	0.54548
Arbutin	-0.16143	0.56544
GA der 1	-0.15767	0.57467
AN der	-0.14553	0.60481
CAT der 5	-0.13116	0.64128
CAT der 1	-0.11239	0.69004
CAT der 8	-0.089378	0.75142
TPC - HPLC	-0.080528	0.77542
CAT der 4	-0.035058	0.90129
CAT der 2	0.025705	0.92755
CAT der 3	-0.0059986	0.98307

Table S5. Statistical data of correlation analyses with IL-6 secretion. TPC = Total phenolic compounds; CAT = Catechin; GA = Gallic acid; HA = Hydroxybenzoic acid; AN = Anthocyanin; der = derivative

	Correlation coefficient	P-value
IL-6	1	0
TPC - Folin	0.69116	0.0043234
DPPH IC50	-0.68601	0.0047467
IL-1β	0.5964	0.018938
CAA IC50	-0.4816	0.069107
TNF-α	0.32242	0.24119
AN der	-0.23216	0.40506
GA der 1	0.1661	0.55411
CAT der 2	0.14817	0.5982
GA der 2	-0.14502	0.60609
TPC - HPLC	0.13518	0.63099
CAT der 3	0.1271	0.65172
CAT der 7	-0.11322	0.68788
CAT der 4	0.10228	0.71682
Gallic acid	-0.096192	0.73309
HA der	-0.095847	0.73401
Arbutin	-0.070914	0.80171
CAT der 6	-0.062288	0.82546
CAT der 5	-0.019558	0.94484
CAT der 8	0.019034	0.94632
CAT der 1	-0.0063611	0.98205

Figure S1. Chromatogram of phenolic compounds from KOM and MHWE extract (λ = 280 nm): 1. Arbutin, 2. Gallic acid derivative (1), 3. Gallic acid derivative (2), 4. Gallic acid, 5. Hydroxybenzoic acid derivative, 6. Catechin derivative (1), 7. Catechin derivative (2), 8. Anthocyanin derivative, 9. Catechin derivative (3), 10. Catechin derivative (4), 11. Catechin derivative (5), 12. Catechin derivative (6), 13. Catechin derivative (7), 14. Catechin derivative (8).