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Microencapsulation of *Theobroma cacao* L polyphenols: A high-value approach with *in vitro* anti-*Trypanosoma cruzi*, immunomodulatory and antioxidant activities

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ABSTRACT

Chagas disease (CHD) is the highest economic burden parasitosis worldwide and the most important cardiac infection, without therapeutic alternatives to halt or reverse its progression. In CHD-experimental models, antioxidant and anti-inflammatory compounds have demonstrated therapeutic potential in cardiac dysfunction. *Theobroma cacao* polyphenols are potent natural antioxidants with cardioprotective and anti-inflammatory action, which are susceptible to degradation, requiring technological approaches to guarantee their protection, stability, and controlled release. Here, 21 cocoa polyphenol-rich microencapsulates were produced by spray-drying and freeze-drying techniques using two wall materials (maltodextrin and gum arabic). Chemical (total and individual phenolic content and antioxidant activity), structural (morphology), and biological parameters (cytotoxicity, trypanocidal, antioxidant, and immunomodulatory activities) were assessed to determine the most efficient microencapsulation conditions on *Trypanosoma cruzi*-infected myocardioblast and macrophage cells. Significant antiproliferative properties against infected cells (superior to benznidazole) were found in two microencapsulates which also exhibited cardioprotective properties against oxidative stress, inflammation, and cell death.

1. Introduction

Chagas disease (CHD) is a neglected tropical infection that is multisystemic and, in cases of late diagnosis and treatment, lifelong [1]. This disease is considered a global public health problem [2], with an estimated prevalence of 6–7 million, an annual incidence of approximately 30,000–40,000, and 12,000 deaths per year [3]. In the chronic phase, this condition is characterized by disabling neurological, cardiac, or digestive clinical manifestations, for which it is considered the most important parasitosis worldwide in terms of morbidity, mortality [1], and economic implications [4].

Chronic Chagasic Cardiomyopathy (CCC) is the most relevant manifestation in the late stage, with variable clinical presentation ranging from the absence of symptoms to myocardial dysfunction and death [5]. The etiopathogenesis of CCC is related to the confluence of cardiac persistence of the parasite, severe immune/inflammatory

Abbreviations: **BENZ:**, Benznidazole; **CCC:**, Chronic chagasic cardiomyopathy; **CHD:**, Chagas disease; **GA:**, Gum arabic; **M:**, Maltodextrin; **PEx:**, Pure extract; **SOD:**, Superoxide dismutase.

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response, autoimmune mechanisms, and constant oxidative stress [5–7]. These factors lead to high amounts of mitochondrial reactive oxygen species (mtROS) production by cardiac cells and inflammatory infiltrated due to membrane mitochondrial disruptions as well as the depletion of mitochondrial antioxidant systems such as manganese-dependent superoxide dismutase (MnSOD) and glutathione peroxidase (GPx) [7]. Likewise, dysregulation of the lymphocyte T-helper (Th)1/Th2 immune response also contributes to this process, establishing an increase in the pro-inflammatory profile of the Th1 [interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)−2, IL-6, IL-9, and IL-12] over Th2 (IL-4, IL-5, IL-10 and IL-13) [6].

Nevertheless, conventional treatments are based on two nitroheterocyclic anti-parasitic agents, benznidazole (BENZ) and nifurtimox. These agents present essential limitations leading to high therapeutic failure percentages. These limitations include low curative efficacy in the chronic phase; ineffectiveness in preventing the progression of cardiomyopathy; multiple side effects; prolonged regimens; and natural and acquired resistance [8]. Currently, there is no effective therapy for controlling CHD and preventing CCC. Therefore, it is pertinent to search for new pharmacological approach. Recently, the potential of antioxidant agents as possible therapies to control the genesis and progression of cardiac affection in the chronic phase of CHD has been extensively studied. In this regard, antioxidants such as selenium derivatives, vitamin C, melatonin, resveratrol, and *Lippia alba* (Carvone chemotype) essential oils have demonstrated benefits in cellular and animal models of CHD [9–12].

Theobroma cacao is one of the most important crops due to its nutritional, pharmacological, and economic significance. In the period 2018/2019, the Colombian cocoa production was 62,200 tons [13,14]. This plant has been described as one of the plants with the highest antioxidant capacity [15] which is attributable to various chemical nuclei including flavonoids, stilbenes, phenolic acids, and amino acid derivatives [16]. In this regard, the principal biological properties of *T. cacao* such as its protective potential against oxidative stress, inflammation, and cardiovascular disease, have been mainly ascribed to its richness in polyphenolic compounds [17]. These agents are reducing factors, hydrogen bond donors, and metal chelators that counteract reactive oxygen species (ROS) by converting them to less dangerous compounds. This neutralization is mediated by alterations in signaling and transcription pathways of the antioxidant systems, as well as innate and acquired immunity [18,19]. The aforementioned characteristics make these metabolites potential prophylactic and therapeutic compounds against systemic inflammation, vascular events, lipid metabolism, and immune disorders, all of which have been related to the genesis of cardiovascular disease.

In *T. cacao*, the three principal groups of polyphenols described are flavan-3-ols (37%), with (-)-epicatechin accounting for up to 35% of polyphenol content; anthocyanins (4%); and proanthocyanidins (58%) [20]. In *in vivo* models of induced myocarditis, these compounds have been shown to ameliorate the inflammatory response by inhibiting nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB)/IL-1β and stimulating the anti-inflammatory cytokines IL-4, IL-5, and IL-10 [21–23]. Similarly, oral cacao bean polyphenols have demonstrated their potential as heart failure therapy by significantly suppressing phenylephrine-induced cardiomyocyte hypertrophy and hypertrophic gene transcription. These effects have been attributed to the inhibition of extracellular signal-regulated kinase 1/2 and GATA binding protein 4 phosphorylation in the cardiomyocytes [24]. Likewise, in mice with experimentally induced myocardial ischemia, an oral dose of flavonol (-)-epicatechin significantly prevented cardiac injury through the PTEN/PI3K/AKT signaling pathway [25]. On the other hand, multiple clinical studies have also provided evidence for the cardiopreventive role of cocoa oral supplementation averting chronic cardiovascular events, lowering blood pressure, improving endothelial dysfunction, suppressing the development of atherosclerotic lesions, and inhibiting atherosclerosis [26,27]. Additionally, cocoa supplementation has been shown to ameliorate lipid and glucose metabolism, inhibit platelet aggregation, and prevent myocardial ischemia-reperfusion damage, cardiac hypertrophy, oxidative stress injury, and coronary occlusion [26,27]. Thus, considering the aforementioned health benefits of cocoa and the importance of inflammation and oxidative stress in the pathogenesis of CCC, it is valid to hypothesize the beneficial potential of cocoa polyphenolic extracts in the therapeutic intervention of chronic CHD.

Since cocoa flavonoids are widely recognized as responsible for major health benefits, and in order to reduce the risk of unexpected pharmacological interactions, in this work 21 flavonoid-rich polyphenols extracts were produced from the nut of *T. cacao*. Thus, two different encapsulating techniques (freeze-drying or spray-dryer) and two wall materials for encapsulation process (gum arabic or maltodextrin) were used at different ratios (8% or 16%). The encapsulating agents were selected owing to their recognized safety, biocompatibility, high solubility, and low viscosity. For spry-drying methodology two different inlet temperatures were evaluated (140°C and 160°C). The microencapsulates were chemically characterized for their composition, antioxidant capability, moisture content, and morphological structure. Additionally, their biological activities were established including cytotoxic, trypanocidal, antioxidant, and anti-inflammatory capacities on *T. cruzi*-infected cells of murine myocardioblasts (H9c2) and macrophages (J774A.1).

The encapsulating technologies proposed herein represent an innovative approach to guarantee high levels of bioactive compounds for pharmaceutical applications (nutraceuticals) [28]. Considering the advantages that these platforms offer in preserving polyphenol bioactivity by shielding the core material from adverse conditions (such as high temperatures, acidic pH, light and oxygen exposure, moisture, and chemical modification of intestinal barriers) to which the beans are exposed during fermentation, drying, roasting, and gastrointestinal absorption [20,29,30]. Likewise, the direct use of polyphenolic-pure compounds as oral supplements is challenging due to their unpalatable flavor and instability. The encapsulation process can improve the organoleptic properties of these extracts [29,30]. Thus, the present study contributes to the development of new products in the cocoa productive chain with higher added value.

2. Materials and methods

2.1. Cell cultures

Murine H9c2 myocardioblasts (ATCC-CRL-1446) and murine macrophages J774A.1 (ATCC- TIB-67) was cultured in Dulbecco's Modified Eagle's medium high in glucose (D-MEM, Gibco, CA, USA), pH: 7.2, added with sodium bicarbonate (Sigma-Aldrich, St. Louis, MO), sodium pyruvate (Sigma-Aldrich), 10% inactive Fetal Bovine Fetal Serum (SFBi, Gibco) and 1% Penicillin-Streptomycin (Gibco); and incubated at 37°C, 5% CO_2 and 95% humidity. Epimastigotes of *T. cruzi* (TcI) strain SYLVIO-X10 were cultured in Liver Infusion Tryptose liquid medium (LIT, Becton Dickinson, FL, USA) supplemented with 10% SFBi and incubated at 28°C. Trypomastigotes were obtained from stationary phase epimastigotes by temperature-induced differentiation.

2.2. Plant material

For this work, only healthy beans (without infection or physical damage) from *T. cacao* (clones CCN51, ICS-1, ICS-65, TSH-565, IMC-67, and ICS-60) were chosen by random sampling in farm "Villa Helena SAS" (4°2804N 75°1150′ W, at an altitude of 1296 m above sea level, mean temperature of 23.2 °C, relative humidity of 80–90%, and 1691 mm of annual precipitation), Department of Tolima (Andean Region, Colombia). The specimens were grown under standard conditions. The cocoa pods were placed in a portable cooler and transported to the laboratory, where they were opened, and the beans immediately had the

mucilage removed, placed in Ziploc bags, and frozen in a freeze-dried BK-FD18PT (Biobase, Jinan-China).

2.3. Samples extraction

The freeze-dried beans were crushed and placed in amber containers to be defatted with hexane. The resulting solids were extracted by cold maceration using aqueous ethanol (70:30) for 3 days with daily agitation. After, the extracts were vacuum filtered and concentrated under reduced pressure (Heidoph Hei-VAP, Schwabach-Germany; Vacuubrand PC3001 VARIOpro, Wertheim-Germany). The aqueous extracts obtained were freeze-dried and stored in amber glass containers at −20°C until they were processed to obtain the encapsulates by spray drying and freeze-drying.

2.4. Encapsulation

A laboratory spray dryer YM-015 (Yuming Instrument Co. Ltd., Shanghai, China) and a vertical freeze dryer were used for spray drying and freeze-drying, respectively. For both, the encapsulating agent was calculated as %w/w to the cocoa extract weight. The extract aqueous fractions were dispersed by magnetic stirring with two concentrations (8% and 16%) of the encapsulating agent (maltodextrin and gum arabic). Spray drying technology was only applied on CCN51 extracts and the liquids obtained were atomized at 140 °C or 160 °C through a peristaltic pump and a 1 mm diameter nozzle. CCN51 and the remaining clone extracts were treated through freeze-drying. The emulsions were poured into 20 mL amber containers and cooled at −60 ºC. Then, the compounds were dried in the equipment trays with a condenser temperature of −80 ºC and 10 Pa of pressure. The moisture of the microcapsules was determined using a moisture analyzer MB90 (AHOUS, México, D.F.-México).

2.5. Chemical characterization by UHPLC-ESI-Orbitrap-HRMS

A Dionex™ Ultimate™ 3000 ultra-high-performance liquid chromatography (UHPLC) equipment (Thermo Scientific, Waltham, MA, USA) was used, coupled with a degasser (SRD-3400), a binary gradient pump (HPG3400RS), an autosampler (WPS 300TRS) and a thermosetting unit for the column (TC 3000), a Hypersil GOLD™ aQ column [100 mm×2.1 mm I.D. x 1, 9 μm particle size] (Thermo Scientific). The column temperature was maintained at 35°C. The mobile phase for the positive ion acquisition mode was a mixture of water (A) with 0.2% formic acid and acetonitrile (B). For negative ion acquisition mode, it was water (A) with 5 mM ammonium form and acetonitrile (B). The following was the mobile phase schedule: 100% (A) linearly changed to 100% (B) in eight minutes, remained constant for four minutes, inverted to 100% (A) for one minute, and remained unchanged for three minutes. A high-resolution mass spectrometry detector with an Orbitrap ion current measurement system (Q Exactive Plus Thermo Scientific) was used, operating in full MS scan mode with a resolution of 70,000. The injection volume was 1 μL; drying gas: N2, 7 L/min; gas nebulizer: N2, 40 psi; capillary voltage: 3.5 kV; mass range m/z 100–1100. Mass spectra were acquired in full scan mode, recording product ions formed in the HCD high-energy dissociation cell (positive mode 10–40 V, negative mode 10–30 V). Compounds were identified based on the elemental composition of the protonated or deprotonated molecules and their product ions. The data obtained were analyzed with the Xcalibur™ v3.1 software (Thermo Scientific).

2.6. Determination of total phenolic content (TPC)

TPC was assessed by UV–Vis spectrophotometry [31] with slight modifications. Thus, 0.25 mL of the extract was mixed with 0.25 mL of the Folin-Ciocalteu reagent (Sigma Aldrich) and 2.0 mL of distilled water. After 3 min at room temperature (25 °C), sodium carbonate 20% w/v (Na_2CO_3, 0.25 mL) solution was added, and the mixture was placed in a water bath for 30 min at 37 °C. Then, absorbance was read at 760 nm in a Cary® 60 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). Gallic acid was used for the calibration curve, and TPC was expressed as milligram equivalent of gallic acid (mg GAE) per gram of dried sample (mg GAE/g dried sample). The measures were taken in triplicate.

2.7. Antioxidant capacity

2.7.1. Ferric reducing antioxidant power assay (FRAP)

FRAP analyses were performed [32] with some modifications. Briefly, 280 μL of diluted extract and 2.1 μL FRAP were mixed and incubated for 4 min at 37 °C. The absorbance was read at 593 nm in a quartz cell in a Cary® 60 UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA). A calibration curve was prepared using different concentrations of Trolox (0.1, 0.2, 0.4, 0.6, and 0.8 mmol/L) and FRAP; data were expressed as milligram equivalent of Trolox (mmolET) per gram of dried sample material (mmolET/g dried sample).

2.7.2. DPPH• scavenging ability

The antioxidant capacity was determined using the DPPH• (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging method [33], with some modifications. An aliquot (50 μL) of the extract, previously diluted, was added to 250 μL of a methanolic solution of DPPH• (0.5 mM) and shaken. After 25 min at 25 °C, the absorbance was measured at 517 nm in an Agilent Cary® 60 UV-Vis Spectrophotometer. A calibration curve was prepared using Trolox (Sigma Aldrich) at different concentrations (20 – 80 μM). Results were expressed as μmol of Trolox equivalent per gram of sample on a dry weight basis (μM TE/g DW).

2.7.3. Oxygen radical absorbance capacity (ORAC) assay

The determination was carried out according to [34], with some modifications. All the reagents and the samples were diluted in a 75 mM phosphate buffer (pH 7.4). Sample (50 μL) and fluorescein (150 μL; 1.6 μM) were added to a microplate well and pre-incubated for 30 min at 37 ºC. Then, a solution of AAPH (50 μL; 125 mM) was added. The fluorescence was recorded every minute for 90 min at excitation and emission wavelengths of 485 and 520 nm, respectively; the microplate was shaken before each read. Fluorescein plus AAPH was used as a blank, replacing the sample with phosphate buffer. Five concentrations of Trolox (12,5–100 μM) were used as reference. The ORAC values were determined by the regression equations between the Trolox concentration and the net area under the fluorescein decay curve and were expressed as μmol of Trolox equivalents per 100 grams (μmol of TE/100 g DW).

2.8. Morphological characterization of the particles-scanning electron microscopy (SEM)

It was performed in a Zeiss EVO HD 15 scanning electron microscope (Zeiss, Oberkochen, Germany), with an EHT (Electron High Tension) of 15 kV. The secondary electron detector (in variable pressure mode) was used to appreciate in detail the topography of the samples with a high depth of field. The samples were treated with the Q150 R ES Plus metallizer by sputtering in the gas phase at a high vacuum (10^{-6} mbar) with non-oxidizing metal (gold). The samples were coated with a fine and uniform thickness.

2.9. Cytotoxic activity on H9c2 and J774A.1 cells

H9c2 (80,000 cells/mL) and J774A.1 (40,000 cells/mL) cells were plated in 96-well flat-bottom plates and incubated for 24 hours at 37°C with 5% CO_2 and 95% humidity until monolayer formation. Concentrations between 7000 and 259 μg/mL of the polyphenolic extracts were added, and cells were incubated again for another 24 hours. To

determine cell viability, the reagent 3-(4,5- dimethylthiazol-2-yl)−2,5-diphenyltetrazole bromide (MTT, Sigma-Aldrich) was added, and cells were incubated for 4 h; after which readings were made in an automatic MultiSkan Sky absorbance microplate reader (Thermo Fisher Scientific, Waltham, MA, USA) at 570 nm. Untreated and BENZ-treated cells were used as negative and positive controls, respectively. Experiments were performed in triplicate at three different times, and data were expressed as Cytotoxic Concentration 50 (CC_{50}). Working solutions were pre-treated in phosphate-buffered saline (PBS) with 2% dimethyl sulfoxide (DMSO, Sigma-Aldrich). Compounds were stored at 4°C and protected from light until use.

2.10. Antiparasitic activity on T. cruzi

For this assay, a monolayer of H9c2 cells (60,000 cells/mL) or J774A.1 macrophage (30,000 cells/mL) was infected with *T. cruzi* trypomastigotes in variable cells: parasite ratio between 1:5–1:10 ratios and incubated for 24 hours at 37°C, 5% CO_2 and 95% humidity until amastigotes appearing. Then, cells were exposed for 24 hours to different concentrations of the polyphenolic extracts (3.7 and 100 μg/mL). Untreated and BENZ-treated cells were used as negative and positive controls, respectively. The antiparasitic activity against *T. cruzi* amastigotes was determined as described previously [35]. Tests were performed in triplicate in three experiments and the results expressed as Inhibitory Concentration 50 (IC_{50}).

2.11. Morpho-physiological changes on H9c2 and J774A.1

These analyses were carried out on infected and uninfected H9c2 and J774A.1 cells exposed for 24 hours to CC_{25} and IC_{50} of the therapies. Phenotypic changes at the nuclear and cytoplasmic levels were examined by optical microscopy through Wright staining (Albor Chemicals, Bogotá, Colombia). Mitochondrial membrane potential ($\Delta\psi M$) by MitoProbe™ JC-1 probe (Invitrogen, CA, USA) was estimated according to the manufacturer's instructions, in a fluorescence microscope (Nikon Eclipse Ni - U, NY, USA) at an excitation/emission wavelength of 585/590 nm.

2.12. Mitochondrial superoxide determination

For this test, the fluorogenic probe MitoSOX™ Red (Invitrogen) was used according to the manufacturer's instructions. Myocardioblast and macrophage cells were treated with the CC_{50} (uninfected) and IC_{50} (infected) of the different compounds. Continuous microscopic fields (equivalent to 200 cells) were observed under a fluorescence microscope (Nikon Eclipse Ni - U) at an excitation/emission wavelength of 510/580 nm. Fluorescence intensity was estimated using Adobe® Photoshop 7.0 software (Adobe Systems Incorporated, San Jose, CA, USA). Untreated or DMSO-treated cells were the negative or positive control, respectively.

2.13. Effect on antioxidant systems of infected myocardioblasts

For this assay, H9c2 cells were arranged in 12-well plates and exposed for 24 hours to the CC_{25} (non-infected) and the IC_{50} (infected) doses of the therapies. Total Superoxide Dismutase (SOD-T) activity was determined using a Total Superoxide Dismutase Activity Assay Kit (T-SOD, MyBioSource, CA, USA); and total (GSH-T), reduced (GSH) and oxidized (GSSG) Glutathione by a Total Glutathione Colorimetric Kit (GSH-T, MyBioSource). All determinations were made following the manufacturer's instructions for sonicated cells.

2.14. Immunomodulatory effect on infected macrophages

The evaluation of the immunomodulatory effect was determined by measuring the levels of proinflammatory (INF-γ) and anti-inflammatory (IL-4 and IL-10) cytokines in the supernatant of infected and uninfected J774A.1 macrophages treated with the CC_{50} of the studied therapies. This assay was performed through the multiplex platform using the Milliplex Map Mouse Cytokine/Chemokine MagneticBead Panel kit (Hcytomag-70k-Merck), under Luminex® xMAP® technology, according to the manufacturer's instructions.

2.15. Statistical analysis

To obtain the different extracts dried by spray drying, a 23-factorial design was used to evaluate the effect of 8% and 16% w/w maltodextrin and gum arabic at 140 ºC or 160 ºC for 8 treatments. A one-way analysis of variance (ANOVA) was applied, complemented by a comparison of means using the Tukey test to evaluate the statistical differences using the Statgraphics Centurion XVIII software (Statgraphics, Madrid, Spain). The CC_{50} and IC_{50} were calculated in XLfit5.5.0.5TM statistical software (IDBS, Boston, MA, USA) by sigmoidal regression from the inhibition percentages. Statistical differences between the mean values of CC_{50} and IC_{50}, fluorescence intensities in oxidative stress assays, and cytokine levels were performed using GraphPad Prism 9.5.1 software (Dotmatics, Boston, MA, USA) through analysis of variance (ANOVA). Dunnett's test was used for multiple comparisons, establishing a 95% confidence level in all cases. Finally, the statistical differences between values of TPC, chemical antioxidant capacity, and humidity percentages of the *T. cacao* microencapsulates and the corresponding pure extract were evaluated using the paired t test. Possible correlations between chemical or biological parameters were studied using the GraphPad Prism software. 9.5.1 (Dotmatics, Boston, MA, USA). A significant correlation was established with values <0.8 and 95% confidence level. All analyses were performed in triplicate on three independent experiments, and measurement data were expressed as the mean ± standard deviation.

3. Results and discussion

For the development of the present study, six cocoa clones CCN51, ICS-95, IMC-67, TSH-565, ICS-60, and ICS-1, were collected. In order to mitigate the impact of roasting processes on the active principles, each of them underwent a process of extracting fresh nuts using a mixture of water:alcohol, followed by freeze-drying. The polyphenol-rich extracts of each clone were subsequently evaluated for their chemical antioxidant capacity and characterized using chromatographic methods coupled to high-resolution mass spectrometry. This allowed us to identify active principles of interest for the development of this research. Note that, only clone CCN51 was microencapsulated using both spray-drying and freeze-drying. This choice was based on a review of the chemical and abundance profiles, which indicated that this clone had the most promising epicatechin content.

3.1. Total phenolic content (TPC)

It is widely recognized that there is a positive relationship between cocoa polyphenol content and cardioprotective effects, which is mainly attributed to their antioxidant flavonoids [17]. However, these biological effects are significantly dependent on their bioavailability and antioxidant status. In this regard, the TPC values were measured in order to determine the losses derived from drying and encapsulating processes, as well as their corresponding effect on polymerization and dilution. Our results showed that the techniques developed herein allow us to obtain TPC values within the ranges previously reported for Colombian clones [36]; with substantial improvements in the polyphenol content for the encapsulations of the clones ICS-60, ICS-1, and CCN51. Significant differences ($p < 0.05$) were observed between the drying methodologies, with higher values in TPC of freeze-drying encapsulated extracts (135.56–66.63 mgEAG/g) than spray drying (64.87–18.05 mgeAG/g) (Table 1). These results were associated with the thermal susceptibility of the polyphenols and the absence of high

Table 1
Total polyphenol content, antioxidant capacity, and moisture percentages of the *T. cacao* microencapsulates.

Clone /Encapsulation Technique	Coated Treatment	TPC± SD (mgEAG/g)	FRAP± SD (mmolET/g)	DPPH± SD (μmolET/g)	ORAC± SD (μmolET/100 g)	Moisture (%)
CCN51	PEx	65±0.9	659±7	251±3	85,785±2378	-
/Spray-dryer^{+}	M8–140	35±0.03*	460±8**	202±2**	68,042±4800*	7.6±0.5
	M8–160	24±0.7**	252±13**	166±1**	49,102±3539*	7.6±0.5
	M16–140	18±0.6**	148±12**	118±3**	73,302±5284	7.4±0.5
	M16–160	22±2.4**	107±3**	102±2**	45,351±907*	7.5±0.4
	A8–140	28±0.9***	537±22*	178±1**	40,451±1849**	10.8±0.2
	A8–160	23±0.5**	268±12**	152±0.3**	34,447±1380**	11.4±0.3
	A16–140	35±11.3	256±2**	145±1.1**	49,254±1935**	11.6±0.3
	A16–160	18±1.2**	187±4**	109±0.4**	28,306±1803**	10.6±0.1
CCN51	PEx	65±0.9	659±7*	251±3	85,785±2378	-
/Freeze-dryer^{++}	CCN51M8	123±4.4*	1163±37	488±5**	129,246±1791**	11.8±0.7
	CCN51M16	120±5.8*	673±14**	479±10**	137,319±1187***	12.4±0.9
	CCN51GA8	126±5.2*	1310 ±3*	482±9**	130,979±2238***	11.8±0.5
	CCN51GA16	119±2.4**	1015±51	488±10**	25,560±1634**	13.1±0.7
ICS-95	ICS-95PEx	81±2	590±4	301±4	56,246±1732	-
/Freeze-dryer	ICS-95M8	72±1*	558±7*	294±4	53,361±1830*	12.7±2.0
	ICS-95M16	67±1*	576±4	292±7	53,765±2129	12.2±1.5
	ICS-95GA8	77±2	583±6	292±3*	55,533±1344	11.6±1.0
	ICS-95GA16	68±3*	437±6**	290±4	51,053±1832**	13.3±2.4
IMC-67	IMC-67PEx	80±2	315±8	325±3	45,789,76±626	-
/Freeze-dryer	IMC-67M8	76±2	310±13	321±2	38,650,99±722****	11.8±1.0
	IMC-67M16	77±3	307±2*	312±8	40,062,38±556****	11.7±1.9
	IMC-67GA8	77±1	318±6	314±2*	54,908,95±1026***	12.9±1.9
	IMC-67GA16	75±4	308±6	309±0,2*	54,908,95±1026***	12.2±1.7
TSH-565	TSH-565PEx	91±3	846±28	346±3	75,757±2547	-
/Freeze-dryer	TSH-565M8	84±2*	831±26*	334±4*	73,633±3148	11.9±2.7
	TSH-565M16	79±1*	839±53	320±1*	72,076±2862*	12.2±2.1
	TSH-565GA8	85±2*	813±4	338±0,1	65,713±4779	12.5±2.2
	TSH-565GA16	79±4*	812±12	341±3	66,437±3097*	12.2±1.6
ICS-60	ICS-60PEx	101±1	938±24	435±3	118,527±3278	-
/Freeze-dryer	ICS-60M8	95±3	934±37	421±3	110,407±2552*	11.9±0.6
	ICS-60M16	96±2	896±33	430±4	109,588±3300***	12.7±1.1
	ICS-60GA8	98±1	855±19*	431±4	96,000±2746**	11.5±0.5
	ICS-60GA16	95±2	893±8	427±4*	105,765±3202**	12.6±1.1
ICS-1	ICS-1PEx	136±6	1856±9	475±2	124,746±3245	-
/Freeze-dryer	ICS-1M8	117±3*	1469±13**	463±0,4*	113,920±3599*	12.5±0.7
	ICS-1M16	113±4*	933±1**	471±4	99,733±2561**	11.9±0.7
	ICS-1GA8	120±4*	999±8***	460±3*	121,819±4131	12.8±0.6
	ICS-1GA16	128±7*	1735±8**	471±3	119,937±2912*	12.5±1.0

+CCN51/Spray dryer extracts encapsulated with maltodextrine at 8% (M8) and 16% (M16) or gum arabic at 8% (A8) and 16% (A16); $^{++}$CCN51, ICS-95, IMC-67, TSH-565, ICS-6, and ICS-1 dried by freeze dryer encapsulated with maltodextrine at 8% (M8) and 16 (M16) or gum arabic at 8% (GA8) and 16% (GA16); PEx: pure extract; TPC: Total phenolic content; FRAP: Ferric Reducing Antioxidant Power Assay; DPPH: radical scavenging activity assessment by 2,2-Diphenyl-1-Picrylhydrazyl; ORAC: Oxygen Radical Absorbance Capacity; values expressed as means ± SD; significant differences between microencapsulates and the corresponding pure extract obtained by t-test; *$p<0.05$; **$p<0.001$ ***$p=0.0001$; ****$p<0.0001$.

temperatures in the encapsulation process through freeze-drying. When the drying temperature increases, lower content is expected [30,37]. For instance, the CCN51 extracts processed by spray dryer showed a reduction of 2.5 times in the TPC mean value compared to the corresponding pure extract (25.3±6.7 vs 65±0.9; $p<0.05$). A reverse effect was found for CCN-51 freeze-dried microencapsulates, in which the polyphenol content was twice that of the pure extract (122±3 vs 65 ±0.9; $p<0.05$) and four times more than spray drying extracts (25.3±6.7 vs 122±3, $p<0.05$). Consistently, in microencapsulates obtained through spray-drying, a lower polyphenol content was observed when 160 ºC was used as the drying temperature (Table 1). Thus, the extracts isolated from the CCN51 clone encapsulated in gum arabic (8 or 16%) or maltodextrin (8% or 16%), presented reductions between 19% and 49% in their polyphenol content when processed at 160°C ($p<0.05$). The most noteworthy change was observed for the encapsulated 16% gum arabic (A16–160), which evidenced TPC values of 35.24±11.31 at 140°C vs. 18.05±1.17 at 160°C ($p<0.05$). Additionally, considerable losses in TPC occur during fermentation (bacterial degradation) and drying (high temperatures) [38]. Therefore, when the grains are freeze-dried, fermentation is avoided, reducing flavonoid polymerization to condensed tannins and making it possible to guarantee a higher polyphenols content [37].

On the other hand, no significant differences in TPC losses were found between the pure extract (PEx) and the extracts after freeze-drying treatments. This indicates good availability with the medium and the absence of a dilution effect caused by the addition of the encapsulating agent in the treatments. However, it is crucial to consider that a higher TPC does not necessarily mean a higher antioxidant activity. Therefore, this type of test must be complemented with cell assays.

3.2. Antioxidant capacity

Since all polyphenols exhibit *in vitro* antioxidant properties their content (TPC) had been reported as adequately associated with antioxidant capacity measured by chemical methods [39]. In this work, we observed as the TPC of the studied polyphenol microencapsulates was significantly correlated with the reducing power evaluated by FRAP (correlation index of 0.83) and DPPH (correlation index of 0.98) methodology, but no with ORAC. Contrariwise, an 81% of association was found between the results from microencapsulates FRAP and ORAC assays (Table 1).

Regarding the effect of the drying technology in the chemical antioxidant performance, a substantial impairment ($p<0.05$) was induced

by high temperatures (spray-drying) processes. Thus, microencapsulates obtained by freeze-drying presented FRAP, DPPH, and ORAC values between two and four times higher than those obtained in extracts processed at high temperature (mean values FRAP, DPPH, and ORAC for freeze dryer extracts of 811±27, 386±5, 860,207±53,589, respectively; vs 277±149; 147±35, 48,531±15,482, respectively for spray dryer extracts) (Table 1). For instance, FRAP values for the spray dryer microencapsulates were between 536.95±21.50 and 107.41±3.21 (μmolET/g), with treatment A8–140 having the highest value for FRAP and M16–160 the lowest value. However, the results reported herein are above those reported in the literature for dark chocolate samples of different origins and cocoa percentages [40,41]. Instead, the reducing power evaluated by FRAP methodology had values between 1735.35 ±8.01 and 307.20±2.32 μmolET/g for the encapsulates obtained by freeze-drying; the cocoa clones with the highest antioxidant capacity in this methodology were ICS-1 and CCN51.

The DPPH radical trapping activity presented values between 487.79 ±9.68 and 289.48±4.37 μmolET/g for the encapsulates obtained by freeze-drying (Table 1); the cocoa clones with the highest antioxidant capacity in this method were ICS-1 and CCN51. In spray dryer they ranged from 201.99±1.83–101.93±2.43 μmolET/g, with M8–140 being the treatment with the highest value and M16–160 the lowest (Table 1). Values within the ranges found have been reported in samples of Colombian cocoa, Brazilian cocoa and chocolates produced in Serbia [41,42].

The values of antioxidant capacity evaluated by the method ORAC were between 119937.34±2912 and 38650.99±722 for the encapsulates obtained by freeze-drying (Table 1); the clones with the highest antioxidant capacity in this methodology were ICS-1 and ICS-60. For spray dryer, they ranged between 73302.38±5284 and 28306.12 ±1803, where the treatment with the highest value was M16–140 and A16–160 the lowest. The ORAC values found in this work are similar to those reported by [36], comparative data found on cocoa beans of clone CCN51 from French Guiana, Carillo, Londoño and Gil [43]), as well as those described in the ORAC database for selected foods of the Department of Agriculture [44].

By the aforementioned methodologies, freeze-drying (Table 1) microencapsulated displayed superior chemical antioxidant capacity without significant differences between the treatments of each clone and the respective crude extract (PEx). In contrast, spray-drying performance was substantially lower than that obtained from freeze-drying and with the initial antioxidant capacity values obtained from the crude pure extracts. This impairment was attributed to the high temperature to which the extract droplets were exposed during the spray-drying process and the isolation suffered by the antioxidant compounds from the environment. Consequently, extracts produced at 160ºC evidenced a lower antioxidant capacity (by FRAP, DPPH, and ORAC) than those at 140ºC. Likewise, the higher antioxidant performance reported herein for freeze-drying microencapsulated may also be attributed to the absence of fermentation. For instance, CCN-51GA18 (obtained by freeze-drying, using gum arabic at 8%) presented the better performance in the three studied chemical antioxidant tests. Therefore, in cocoa derivates production for functional purposes, freeze-drying should preferably be used as a drying method to maintain the content of antioxidant compounds in their monomeric form and with a lower degree of polymerization, which is associated with more significant bioactivity [37].

In this study, dark chocolate, cocoa powder, and cocoa encapsulates had ORAC values above 40,000 μmolET/100 g, which places these products within the top 10 foods with the greatest antioxidant capacity according to the ORAC methodology. These results represent an interesting opportunity for developing new products aimed at the functional market, especially with cocoa clones, such as TSH-565, ICS-1, and CCN51 that are widely cultivated in Colombia and have a high content of cocoa.

The percentage of moisture in the encapsulates obtained by freeze-drying was determined to be between 11.45±0.54% and 13.31 ±2.36%, while the results for the spray-dried microencapsulates varied between 7.42±0.48% and 11.37±0.27% ($p<0.05$) (Table 1). Different fruit and plant extracts have been encapsulated by spray drying, achieving low moisture values (<10%) [45], which avoids modifications such as agglomeration, browning, collapse, and oxidation during storage. The treatments with higher humidity were those using gum arabic ($p<0.05$), in both spray-drying and freeze-drying microencapsulates. This could be explained by the fact that water experiences greater difficulty diffusing on the surface of this polymer compared to maltodextrin, causing a higher final humidity. It has been reported that higher moisture in microencapsulates compromises microbiological stability, which negatively affects the functional properties over time, as well as making the product more difficult to handle [45]. These findings of chemical antioxidant activity confirm the importance of innovation in the extraction processes of cocoa polyphenols (reducing the roasting processes) to mitigate the multiple problems associated with polyphenol polymerization. These beneficial properties can be special for the development of nutraceutical products based on technological innovation that can be applied in the field of medicine [46].

3.3. UHPLC and Orbitrap high-resolution mass spectrometry analysis

Chromatographic analysis of cocoa microencapsulates using the positive mode [UHPLC-ESI(+)-Orbitrap] identified nine polyphenolic compounds derived from flavonoids [one procyanidin C trimer (5), two procyanidin B dimers (3 and 7), and five glycosylated flavonoids (1, 2, 6, 8, and 9) and one epicatechin (4)] [16,20]. Table 2 shows the identification and relative amounts of cocoa clones. The chromatographic profiles of the identified compounds are shown in the supporting data (Table 2; Supplementary material, Figure S1).

Using the negative mode (UHPLC-ESI(-)-Orbitrap) were identified 16 compounds (Supplementary material, Figure S2), seven of them confirmed by standards; and nine tentatively through comparison of the exact masses of the deprotonated molecules and their product-ions with those previously reported [16]. Thus, were identified: flavones [luteolin pentoside, luteolin-hexoside and luteolin (4, 8 and 13)]; procyanidin [C, B1, and B2 (2, 3, and 6)]; flavanols [catechin and epicatechin (1 and 5)]; flavanones [pinocembrin, naranginenia, and eriodictyol (11, 12, and 15)]; and flavonols [rutin and quercetin-pentoside (9 and 10)] (Table 2; Fig. 1).

Epicatechin had the largest chromatographic area in the analyzed cocoa extracts, indicating that this active principle is the majority in the extracts of the analyzed clones. Extracts isolated from the ICS-1 clone were the only ones that reported low percentages of rutin, a very important flavonoid due to its multiple reported bioactivities (Table 2). A significant variation in the flavanols percentage was observed in the diverse clones. Thus, CCN51 demonstrated the highest flavanols percentages (90%), followed by TSH-565 (83%), IMC-67 (80%), ICS-60 (79%), ICS-1 (66%), and ICS-95 (61%). These variations were also presented for other polyphenol groups, possibly related to genetic more than edaphoclimatic conditions [36]. This since the studied clones were collected from the same farm, at the same season, and under the same extraction conditions.

3.4. Morphological characterization of microencapsulates by Scanning electron micrographs (SEM)

SEM assays were performed to assess the size and form details of the encapsulated structures. Both encapsulating materials used (maltodextrin and gum arabic) presented a spherical shape that varied in diameter. Irregularities were observed on the surface of the spheres in spray-drying, and these morphological aspects were maintained even under different heat treatments and concentrations of each encapsulating agent used (Fig. 2). These irregularities may be caused by the high vapor pressure generated, which leads to the breakage of the wall. Thus, when

Table 2
Compounds identified by UHPLC-ESI(-)/(+)-Orbitrap-HRMS in hydroalcoholic extracts from cocoa beans.

N°	Retention Time (min)	Compound*	Formula	[M-H]-/ +m/z	Accuracy (ppm)	MS^2 Ions (ppm)	Chromatography area [peak area count]					
							ICS1	ICS95	CCN51	ICS60	TSH565	IMC67
UHPLC-ESI(-)-Orbitrap-HRMS												
1	3.77	Catechin	$C_{15}H_{14}O_6$	289.7159	0,57	245,08150, 137,02319, 125,02313, 109,02814	2,91E+07	6,79E+06	3,86E+07	2,62E+07	2,05E+07	2,37E+07
2	3.9	Procyanidin B2	$C_{30}H_{26}O_{12}$	577.13452	1.08	407,07693, 289,07159, 245,08150, 125,02313	3,11E+07	2,69E+07	4,03E+07	7,84E+07	5,76E+07	4,39E+07
3	4.13	Procyanidin C isomer	$C_{45}H_{38}O_{18}$	865.19824	0.92	577,13458, 407,07678, 289,07180, 125,02300,	2,23E+07	1,17E+07	1,31E+07	3,73E+07	2,12E+07	2,00E+07
4	4.31	Luteolin-pentoside	$C_{20}H_{20}O_{11}$	435.0929	1.6	285,04022	3,31E+06	1,75E+06	3,66E+06	1,49E+06	1,26E+06	1,46E+06
5	4.35	Epicatechin	$C_{15}H_{14}O_6$	289.0715	0.89	245,08136, 137,02310, 125,02306, 109,02808	5,77E+08	1,82E+08	1,10E+09	9,11E+08	6,40E+08	4,78E+08
6	4.83	Procyanidin B1	$C_{30}H_{26}O_{12}$	577.13477	1.23	407,07700, 289,07166, 245,08151, 125,02316	1,45E+07	8,07E+06	1,54E+07	2,88E+07	1,61E+07	1,69E+07
7	4.89	Procyanidin C isomer	$C_{45}H_{38}O_{18}$	865.19812	0.78	577,13477, 407,07709, 289,07180, 125,02295	5,55E+06	2,71E+06	2,23E+06	5,09E+06	2,66E+06	3,18E+06
8	5.33	Luteolin-hexoside	$C_{21}H_{20}O_{11}$	447.09283	1.00	417,08282, 285,04028	6,99E+06	1,82E+06	1,18E+06	3,61E+06	2,29E+05	7,26E+05
9	5.44	Rutin	$C_{27}H_{30}O_{16}$	609.14569	1.1	463,08820, 301,03513	3,06E+05			-	-	-
10	5.58	Quercetin-pentoside	$C_{20}H_{18}O_{11}$	433.07721	0.98	301,03445	2,36E+07	1,37E+07	1,63E+07	1,41E+07	9,96E+06	1,53E+07
11	5.87	Eriodictyol	$C_{15}H_{12}O_6$	287.05582	1.02	151,00259, 135,04398 107,01252	1,97E+07	6,60E+06	6,25E+06	1,04E+07	4,46E+06	3,77E+06
12	6.36	Naringenin	$C_{15}H_{12}O_5$	271.06088	1.15	151,00258	2,96E+06	1,32E+06	1,42E+06	1,18E+06	1,08E+06	1,15E+06
13	6.44	Luteolin	$C_{15}H_{10}O_6$	285.04031	1.16	199,03928, 151,00258, 133,02831	1,01E+08	2,24E+07	1,36E+07	2,43E+07	7,43E+06	7,08E+06
14	6.8	Flavone	$C_{15}H_{10}O_5$	269.04532	0.84	251.03493, 223,03946	4,12E+07	1,01E+07	7,41E+06	1,73E+07	4,55E+06	3,56E+06
15	7.32	Pinocembrin	$C_{15}H_{12}O_4$	255.06587	1.60	151,00253	1,22E+07	5,01E+06	4,09E+06	6,59E+06	2,39E+06	1,42E+06
16	7.65	Flavone	$C_{15}H_{10}O_5$	269.04547	0.28	239,03464, 213,05501	2,88E+07	8,20E+06	5,48E+06	1,36E+07	4,81E+06	3,65E+06
UHPLC-ESI(+)-Orbitrap-HRMS												
1	2.96	Luteolin-hexoside	$C_{21}H_{20}O_{11}$	449.10828	0.97	287.05478	7,46E+07	6,41E+07	9,44E+07	5,36E+07	3,43E+07	5,24E+07
2	3.07	Luteolin-pentoside	$C_{20}H_{18}O_{10}$	419.09769	0.98	287.05505	2,11E+08	1,41E+08	1,84E+08	2,11E+08	1,32E+08	1,48E+08
3	3.16	Procyanidin B isomer	$C_{30}H_{26}O_{12}$	579.15021	0.87	291.08633	9,92E+07	5,46E+07	1,12E+08	1,89E+08	1,42E+08	8,75E+07
4	3.28	Epicatechin	$C_{15}H_{14}O_6$	291.08646	0.48	165.05481, 139.03914, 123.04436	4,48E+08	1,24E+09	8,01E+08	6,34E+08	5,65E+08	3,43E+08
5	3.32	Procyanidin C isomer	$C_{45}H_{38}O_{18}$	867.21381	0.82	579.15027, 291.08646	4,23E+07	3,19E+07	3,57E+07	7,04E+07	5,51E+07	4,26E+07
6	3.59	Apigenin-hexoside	$C_{21}H_{20}O_{10}$	433.11334	0.96	271.06015	5,16E+06	1,07E+06	1,94E+06	1,72E+06	1,26E+06	1,36E+06
7	3.60	Procyanidin B isomer	$C_{30}H_{26}O_{12}$	579.14954	0.29	291.08633	2,86E+07	1,38E+07	2,50E+07	4,45E+07	2,80E+07	2,20E+07
8	3.64	Quercetin-hexoside	$C_{21}H_{20}O_{12}$	465.10272	0.06	303.04999	1,17E+07	1,03E+07	1,30E+07	1,06E+07	9,66E+06	1,17E+07
9	3.77	Quercetin-pentoside	$C_{20}H_{18}O_{11}$	435.09225	0.43	303.05008	1,31E+07	1,40E+07	1,64E+07	1,53E+07	1,06E+07	1,30E+07

* Presumptive identification based on the comparison of the exact masses of the deprotonated molecules and their product ions with the previously described [16,47,48].

lower drying temperatures were used, most of the water managed to escape through the bark by diffusion, avoiding the breakage of the wall [49]. Consequently, compounds encapsulated by spray-drying evidenced better protection and bioavailability (more controlled release and greater surface area) [29].

Unlike the spherical morphology, the encapsulates obtained by

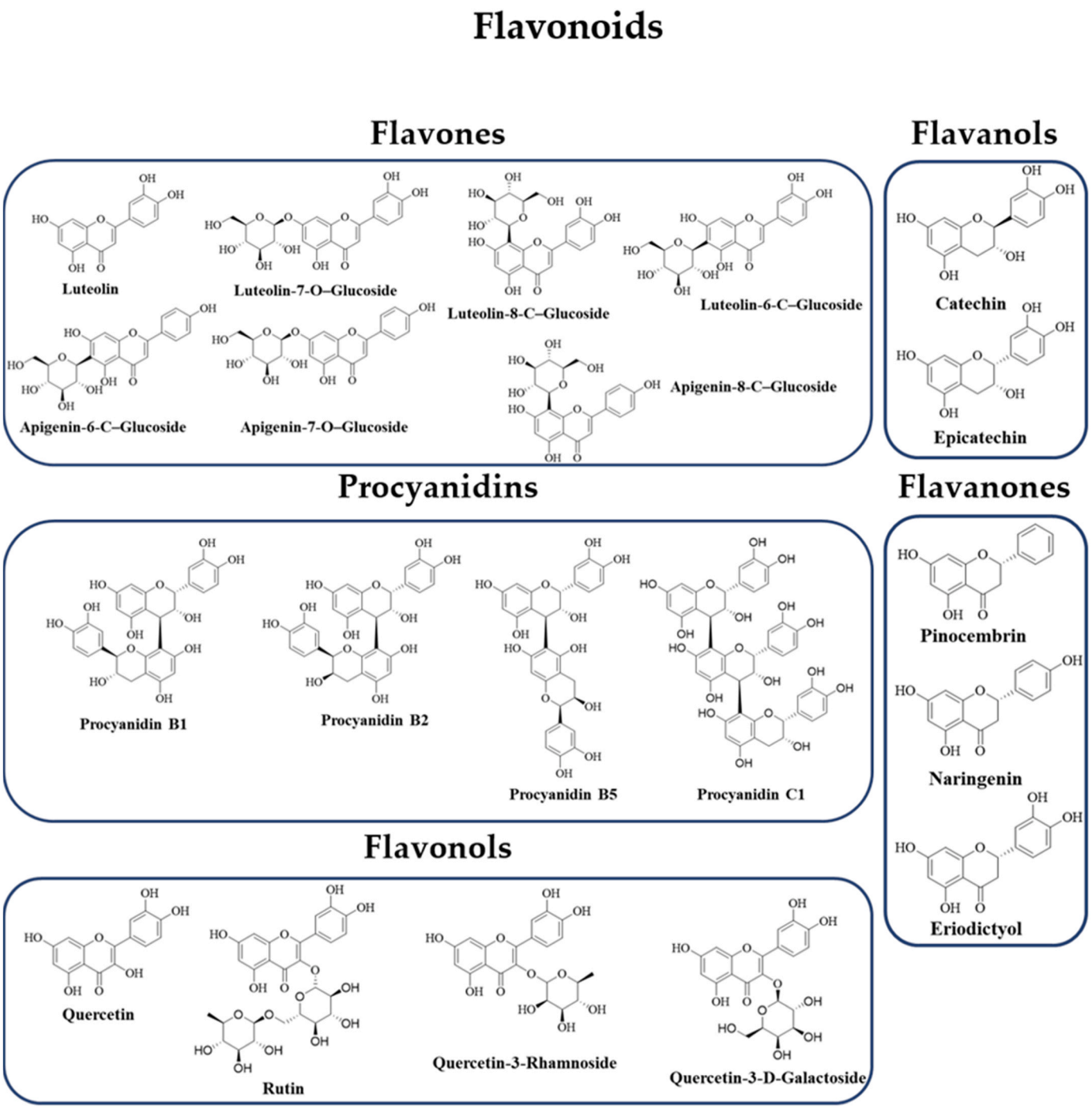


Fig. 1. Structure of compounds identified in *T. cacao* clones.

lyophilization had an amorphous and porous geometry (Fig. 2). This physical characteristic represents a disadvantage associated with the larger exposed area, which can lead to less protection during storage and uncontrolled release of the active principles compared to those obtained by spray-drying. These results are similar to those previously described using the same encapsulation technique and gum arabic and maltodextrin as wall materials [50].

3.5. Cytotoxicity on myocardioblasts (H9c2) and macrophages (J774A.1) cells

On murine myocardioblasts (H9c2), all the polyphenolic extracts studied presented an excellent safety profile, with very high CC_{50} values (CC_{50}>899 μg/mL; and >7000 μg/mL, for the PEx). These values were similar to those obtained for vitamin C (CC_{50} = 5684 μg/mL). BENZ presented cytotoxicity between 4 and 30 times higher than cocoa derivatives (CC_{50}= 236 μg/mL, p<0.0001) (Table 2, Fig. 3). The PEx and the five extracts with the lowest cytotoxicity on cardiac cells (A16–160, A16–140, A8–140, ICS 95-GA, and ICS 95-M) were also tested on murine macrophages J774A.1, finding a CC_{50} > 5000 μg/mL; with PEx being the least cytotoxic compound (CC_{50}> 7000 μg/mL) (Fig. 3). These values are congruent with previous reports in which natural polyphenols (i.e., curcumin, grape, resveratrol, and cherry) and commercial polyphenols (i.e., catechin, epicatechin, epigallocatechin, and proanthocatechin) and microencapsulated in natural polymers, also showed low to no toxicity on mammal cells [10–12].

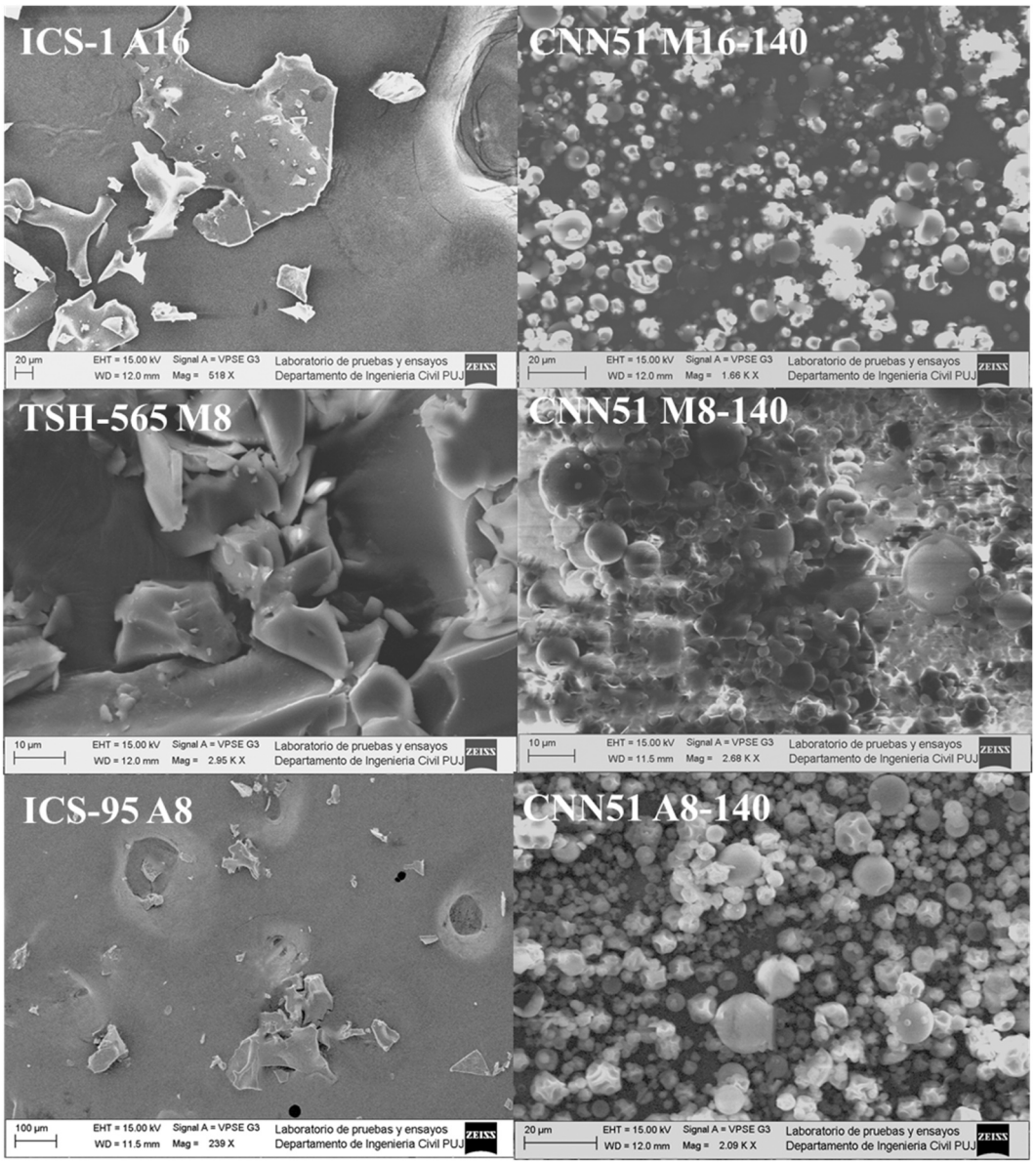


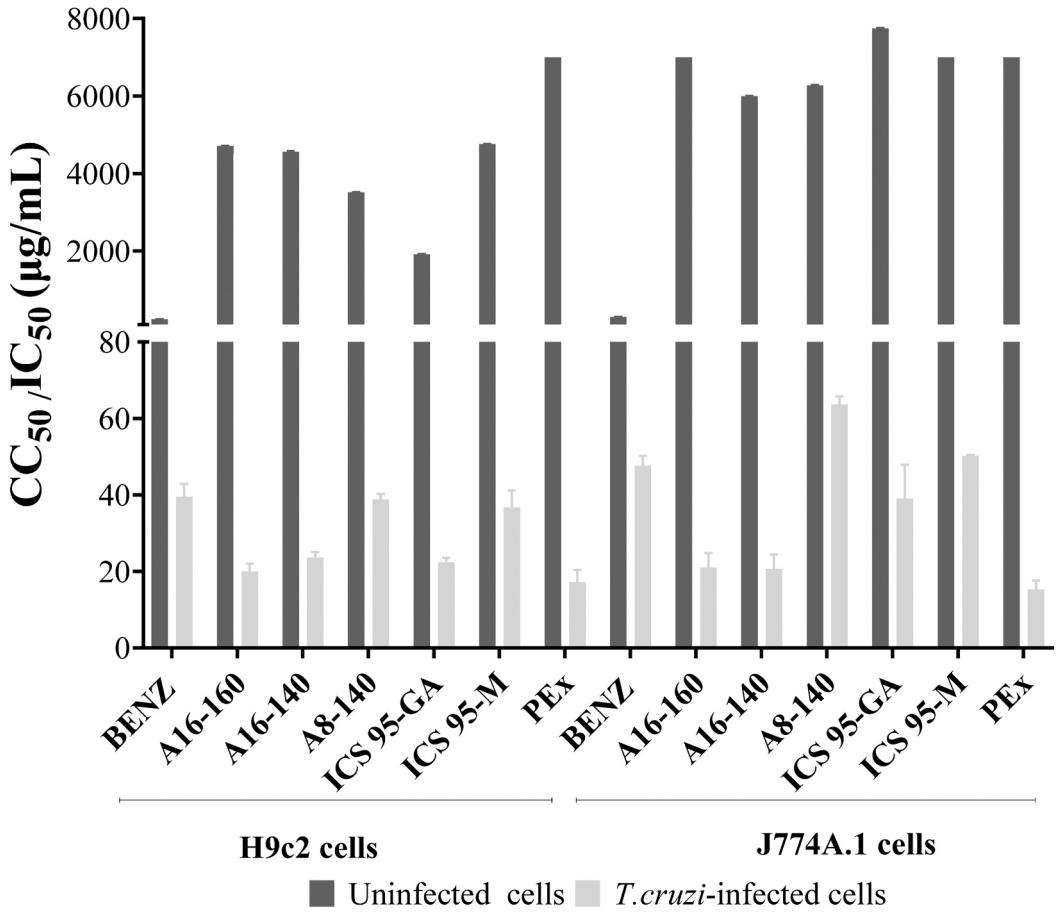
Fig. 2. Scanning electron microscopy of polyphenol extracts obtained under different encapsulants, heat treatments, and cocoa clones. Extracts dried by spray-drying (CCN51) or freeze-drying (ICS-1, TSH-595, and ICS-95), encapsulated with maltodextrine at 8% (M8) and 16 (M16) or gum arabic at 8% (GA8) and 16% (GA16); processed under different temperatures 140°C (140) or 160°C (160).

3.6. Morpho-physiological effect on myocardioblasts (H9c2) and J774 A.1 macrophages cells

Optical and fluorescence microscopy techniques evaluated the cellular effects of the therapies. Untreated (control) H9c2 and J774A.1 cells showed typical morpho-physiological characteristics with cell membrane continuity and preservation of mitochondrial membrane potential (yellow to orange staining by JC-1 probe), as well as minimal levels of mitochondrial superoxide ion (slight red staining by MitoSOX™ Red) (Fig. 4). Cardiac cells also preserved the normal pattern of adhesion and shape.

Further, high toxicity was evidenced on myocardioblasts and macrophages treated with the CC_{50} of BENZ. These cells showed massive lysis, cellular swelling, karyolysis, and a total absence of mitochondrial membrane potential (green staining by JC-1). Likewise, these cells presented high mitochondrial oxidative stress, which was substantially different from untreated cells ($p < 0.01$ for H9c2; and $p < 0.0001$ for J774A.1) (Fig. 4). In addition, cardiac cells subjected to this treatment also showed de-adhesion.

A comparable phenotype to the control was observed in myocardioblasts exposed to the CC_{50} of PEx and the cocoa microencapsulated obtained by spray-dryer and coated with gum arabic (A16–160, A16–140, and A8–140). These compounds were extracted from the same cacao variety, CCN51, which presented the highest catechin and epicatechin concentrations. Among these encapsulated, A16–160 produced the slightest alteration of the cellular phenotype. At the same time, A8–140 was the only spray-dried compound that caused complete loss of mitochondrial membrane potential, as well as significant mitochondrial oxidative stress (compared to the negative control, $p<0.05$), loss of cell membrane continuity, increased cytoplasmic volume, and alterations of genomic material (karyorrhexis) (Fig. 4A). Additionally, this compound presented high agglutination to the cells (low solubility of the extract in the solution and low distribution in the culture), making its observation and technical handling difficult (data did not show). Likewise, on macrophages, the spray-dried microencapsulates (as well as PEx) showed more remarkable preservation of the



Clone /Encapsulation Technique	Extract	H9c2					J774A.1				
		CC_{50} ± SD (µg/mL)	*p*	IC_{50} ± SD (µg/mL)	*p*	SI	CC_{50} ± SD (µg/mL)	*p*	IC_{50} ± SD (µg/mL)	*p*	SI
CCN51 /Spray dryer[&]	A16-160	4,708 ± 7	<0.0001	20 ± 2	<0.0001	235	> 7,000	<0.0001	21 ± 4	<0.0001	>333
	A16-140	4,563 ± 15	<0.0001	24 ±1	<0.0001	190	5,997 ± 7	<0.0001	21 ± 4	<0.0001	285
	A8-140	3,516 ± 5	<0.0001	39 ±1	NS	90	6,273 ± 12	<0.0001	64 ± 2	0.0009	98
	A8-160	5,095 ± 5	<0.0001	-	-	-	-	-	-	-	-
	M16-140	U*	U*	-	-	-	-	-	-	-	-
	M16-160	U*	U*	-	-	-	-	-	-	-	-
	M8-160	4,615 ± 7	<0.0001	-	-	-	-	-	-	-	-
	M8-140	U*	U*	-	-	-	-	-	-	-	-
CCN51 /Freeze dryer+	CCN51GA	899 ± 8	0.0083	-	-	-	-	-	-	-	-
	CCN51M	2,269 ± 29	<0.0001	-	-	-	-	-	-	-	-
ICS-95 / Freeze dryer+	ICS95-GA	1,916 ± 4	<0.0001	22 ±1	<0.0001	87	7,750 ± 4	<0.0001	38 ± 9	0.0495	198
	ICS95-M	4,759 ± 4	<0.0001	37 ± 4	NS	128	>7,000	<0.0001	50 ± 0	NS	140
TSH-565 /Freeze dryer+	TSH-565GA	1,963 ± 1	<0.0001	-	-	-	-	-	-	-	-
	TSH-565M	TSH-M 16	> 7,000	<0.0001	-	-	-	-	-	-	-
IMC-67 /Freeze dryer+	IMC-67GA	2,197 ± 12	<0.0001	-	-	-	-	-	-	-	-
	IMC-67M	2,660 ± 5	<0.0001	-	-	-	-	-	-	-	-
ICS-60 /Freeze dryer+	ICS-60GA8	2,406 ± 14	<0.0001	-	-	-	-	-	-	-	-
	ICS-60M	> 7,000	<0.0001	-	-	-	-	-	-	-	-
ICS-1 /Freeze dryer+	ICS-1GA8	3,165 ± 8	<0.0001	-	-	-	-	-	-	-	-
	ICS-1M8	2,093 ± 23	<0.0001	-	-	-	-	-	-		-
Reference compounds	PEx	>7,000	<0.0001	17 ± 3	<0.0001	411	>7,000	<0.0001	15 ± 2	<0.0001	466
	Vitamin C	5,684 ± 109	<0.0001	-	-	-	-	-	-	-	-
	BENZ	236 ± 4.24	REF	40 ±3	REF	236 ± 4	298 ± 4	REF	48 ± 3	REF	6

Fig. 3. Cytotoxicity and trypanocidal activities of microencapsulates of *T. cacao* on murine myocardioblasts (H9c2) and macrophages (J774 A.1). CC_{50}: Cytotoxic concentration 50; IC_{50}: Inhibitory concentration 50; SD: Standard deviation; SI: Selectivity index; BENZ: benznidazole; PEx: crude pure extract. [&]CCN51/Spray dryer extracts encapsulated with maltodextrine at 8% (M8) and 16% (M16) or gum arabic at 8% (A8) and 16% (A16), processed under inlet temperatures of 140°C (140) or 160°C (160); [+]CCN51, ICS-95, IMC-67, TSH-565, ICS-60, and ICS-1 dried by freeze dryer encapsulated with maltodextrine at 8% (M8) and 16 (M) or gum arabic at 8% (GA8) and 16% (GA);.

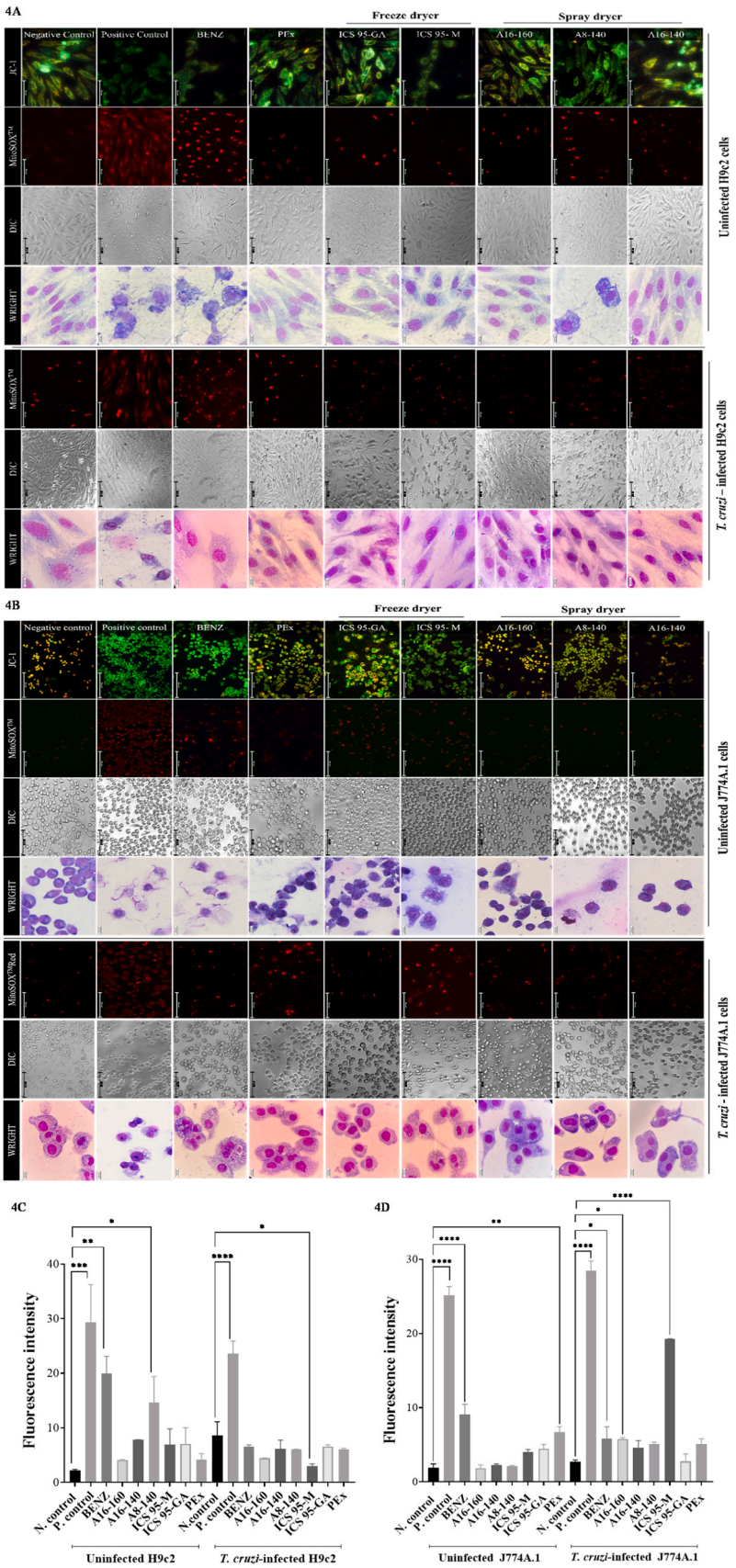


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Fig. 4. Morpho-physiological changes by microencapsulates of *T. cacao* on (A) murine myocardioblasts (H9c2) and (B) macrophages (J774A.1). C) MitoSOX™ Red probe fluorescence intensity on H9c2 and (D) J774A.1 cells. BENZ: benznidazole; PEx: crude pure extract. N. control: untreated cells; P. control: cells treated with dimethyl sulfoxide for MitoSox™ Red, and CCCP (carbonylcyanide-m-chlorophenylhydrazone) for JC-1 assays. A16-140 and A16-160: CCN51/Spray dried extracts encapsulated with gum arabic at 16% processed at 140°C or 160°C, respectively; ICS95-GA and ICS95-M: ICS-95/freeze-dried extracts encapsulated with gum arabic at 16%; tests performed with Cytotoxic Concentrations 50 (CC_{50}); * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

morpho-physiological characteristics (Fig. 4B).

About freeze-dried extracts in myocardioblasts, all microencapsulates caused partial disruption in the mitochondria membrane potential (Fig. 4A). ICS 95-GA was the less toxic compound with the most negligible effect on mitochondria, preserving the other morpho-physiological characteristics (similar to H9c2 untreated control). On the other hand, in macrophages, the involvement of mitochondrial membrane potential was variable. On this point, complete maintenance was observed by the encapsulated in gum arabic (ICS 95-GA), while a total disruption was given by maltodextrin (ICS 95-M). In these same cells, the lyophilized compounds caused nuclear alterations such as karyorrhexis (ICS 95-GA) and karyopyknosis (ICS 95-GA and ICS 95-M). Cytoplasmic vacuoles in macrophages were also observed after ICS 95-M therapy.

3.7. Trypanocidal activity

The ability to inhibit the *T. cruzi*-amastigotas growth was assessed on infected myocardioblasts (H9c2) and macrophages (J774A.1) exposed to variable concentrations of PEx or the five extracts with the lowest cytotoxicity on H9c2 (A16–160, A16–140, on −140, ICS 95-GA, and ICS 95-M). The other encapsulates were discarded for further bioactivity tests considering either the presentation of technical inconveniences or the induction of major cell damage (excessive oxidative stress, cell lysis, and affectation of the mitochondrial membrane potential). For instance, CCN51 clone-derived extracts encapsulated with maltodextrin 8% presented very low solubility preventing their use in working solutions. Regarding to cytotoxicity, all extracts derived from clones ICS-1, ICS-60, TSH-565, CCN51, and IMC-67, freeze-dried obtained, and encapsulated in maltodextrin 8% or 16%, showed the highest affectation of morpho-physiological characteristics (Supplementary material, Figure S3). These findings are in agreement with previous studies that identified the low emulsifying capacity with other materials as the main disadvantage of maltodextrins, in their usage as microencapsulating agents [51].

A significant trypanocidal effect (IC_{50} < 100 μg/mL) was observed for the studied six cocoa extracts on both infected cell models (Fig. 3). All microencapsulates (except ICS 95-M) showed even superior antiparasitic capability than the reference drug, BENZ (IC_{50} H9c2 = 40 μg/mL; IC_{50} J774A.1 = 48 μg/mL, p =0.0001) (Fig. 3). The best trypanocidal performance was exhibited by PEx (IC_{50} of 17 μg/mL and 15 μg/mL, on H9c2 and J774A.1 cells, respectively) and A16–160 (from CCN-51 clone, coated with 16% gum arabic and spray-dried at 160°C) which demonstrated IC_{50} H9c2 = 20 μg/mL (p =< 0.0001); and IC_{50} J774A.1 = 21 μg/mL (p = <0.0001). Also, A16–160 was the most selective treatment on cardiac and macrophage cells (CC_{50} H9c2 > 4708, SI >235; and CC_{50} J774 A.1 >7000, SI >333). In this regard, the capability of the microencapsulates to eliminate the intracellular *T. cruzi* parasites was found to be positively correlated with the (+)-epicatechin content (correlation index of 0.8). For instance, the better trypanocidal performance was evidenced in extracts with the major concentrations of this polyphenol such as the CCN51 pure extract and A16–160 (from CCN51 clone produced by spray-drying) and ICS 95-GA (freeze-drying produced from ICS-95 clone) which presented 1.10E+9 chromatographic areas and 1.82E+8 chromatographic areas for (+)-epicatechin, respectively (Table 2). The other compounds present in the mixture of the studied microencapsulates do not have a significant association with antiparasitic action. These results are in agree with previous studies that reported a significant antiparasitic activity for catechin polyphenols inhibiting the growth and pathogenic mechanisms of common human parasites such as *Giardia*, *Entamoeba*, *Plasmodium*, *Trypanosoma*, *Leishmania* *Teladorsagia*, *Trichostrongylus*, *Ascaris* and *Haemonchus* [52]. Likewise, flavonoids as epicatechin were found to be active against *E. histolytica* (by interference in the function of cytoskeleton proteins) [53] and extracellular kinetoplastids such as *T. brucei* and *L. amazonesis*, acting as a competitive inhibitor of the parasitic arginase [54]. However, to the best of our knowledge this is the first report stablishing the potential of epicatechins as inhibitors of intracellular forms (amastigotes) of *T. cruzi.*

Interestingly, the moisture percentage was also found to be positively correlated with the trypanocidal effect (0.98). Conversely, low values of FRAP activity were associated with better inhibitory performance against *T. cruzi* cells (0.97). ORAC or DPPH analysis did not evidence any association with the antiparasitic effect. These results show the importance of carrying out complementary antioxidant assays on cellular or animal models in order to accurately assess the antioxidant properties of phenolic compounds.

3.8. Morpho-physiological effect on T. cruzi-infected myocardioblasts (H9c2) and J774 A.1 macrophages cells

Infection of H9c2 and J774 A.1 cells by intracellular forms of *T. cruzi* induced cellular swelling, mitochondrial superoxide overproduction (relative to the uninfected control, $p < 0.01$), cytoplasm vacuolization (macrophages), and de-adhesion (myocardioblast). However, no changes in mitochondrial membrane potential were caused by parasite invasion (Fig. 4A and B).

In both infected cell models, exposure to BENZ caused apparent necrotic phenotypic characteristics, including plasma membrane discontinuity and karyolysis (Wright) (Fig. 4A and B). De-adhesion was also observed in cardiac cells treated with this drug (Fig. 4A). In contrast, exposure of infected cells to cocoa extracts (as well as to PEx) triggered slight morphological alterations (compared to untreated control), preserving the plasma membrane integrity. Minimal nuclear changes were observed in macrophages treated with ICS 95-M (karyorrhexis) and ICS 95-GA or A16–160 (karyopicnosis); while cellular aggregation was detected in macrophages exposed to A8–140 (Fig. 4B).

Regarding mitochondrial superoxide levels, parasitized myocardioblasts exposed to all treatments maintained the basal mitochondrial oxidative stress observed for infected but untreated cells. ICS 95-M was the only therapy that caused a significant depletion of mtROS ($p<0.0152$) (Fig. 4A and C). On infected macrophages, BENZ ($p<0.05$), A16–160 ($p<0.05$), and ICS 95-M ($p<0.0001$) caused a substantial increase in mtROS; while A16–140 and ICS 95-GA maintained the basal mitochondrial oxidative stress described in infected cells without treatment (Fig. 4B and D).

3.9. Effect on the activity of antioxidant enzyme systems in murine myocardioblasts

This effect was assessed by measuring the SOD activity, an antioxidant enzymatic system that catalyzes the reduction of superoxide ions. To achieve this, therapies with the best trypanocidal performance were tested on both infected and uninfected myocardioblast. In these cells, *T. cruzi* infection caused a significant impairment of total SOD (SOD-T) activity (827 U/mL vs. 1393 U/mL, $p = 0.0044$). This enzymatic activity was not restored by any therapies in the parasitized cells (except ICS 95-GA) (Fig. 5A). Interestingly, cocoa extracts and BENZ significantly increased SOD-T activity in the non-infected model. The only exception was PEx (1368 U/mL), which maintained the activity of this enzyme at basal levels exhibited by uninfected and untreated cells (control).

A16–160 (from CCN-51 clone spray-dried at 160°C and encapsulating in 16% gum arabic) was the compound with the major antioxidant potential showing up to six times the SOD-T activity observed in control cells (8341 U/mL, $p<0.0001$). These results were significantly higher than those induced by BENZ (5237 U/mL, $p<0.0001$) (Fig. 5A).

The cellular antioxidant potential of the therapies was also evaluated by assessing their effect on GSH levels. This molecule is a significant defensive contributor in maintaining the intracellular redox state through ROS scavenging, and its depletion is a hallmark indicator of oxidative stress. In this regard, healthy myocardioblasts showed a significant increase in GSH ($p<0.05$) after all therapies except PEx (Fig. 5B). Exposure of cells to microencapsulated ICS 95-GA gave a far superior stimulus compared to other treatments with a GSH/GSSG ratio that was 8.7-fold higher than that of non-*T. cruzi*-infected control (25.4 vs. 2.9, $p<0.0001$). On the other hand, *T. cruzi* infection of cardiac cells did not cause noteworthy changes in the GSH/GSSG ratio (2.9 vs. 4.6, $p=0.064$).

Interestingly, the antioxidant effect of ICS 95-GA (from ICS-95 clone obtained by freeze-drying and encapsulated in gum arabic at 16%) was shown to be highly selective for healthy cells. Thus, a significant difference between GHS/GSSG levels was observed between uninfected and infected myocardioblasts treated with this encapsulation (25.4 vs. 4.6, $p<0.0001$). These results allow us to infer a possible cardioprotective effect from the two studied cacao microencapsulates, ICS 95-GA and A16–160 (Fig. 5B).

The impact of *T. cacao* microencapsulates described herein improving the cell-reduced status by elevation of SOD-T and GSH values, can be associated with its major polyphenol, epicatechin. The proposed mechanisms behind the antioxidant activity of this compound are [21,55]: i) ROS reduction capability; ii) ferric ions chelation involved in lipoperoxidation; iii) positive regulation of nuclear factor erythroid 2-related factor 2 (Nrf2), which participates in the regulation of antioxidant proteins; iv) improvement of reduced glutathione levels by potentiating of antioxidant enzymatic systems such as glutathione peroxidase (GPx), glutathione reductase (GR), and SOD1; v) induction of AKT (protein kinase B) and ERK (extracellular signal-regulated kinase) pathways; vii) activation of the phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) pathway in response to transcription factors modulated by oxidative stress, such as NF-κB, activator protein-1 (AP-1), and Nrf2.

3.10. Pro- and anti-inflammatory cytokines levels in vitro

The balance between anti-inflammatory and pro-inflammatory cytokines is critical in determining whether *T. cruzi* will be eliminated without causing further tissue damage involved in the CCC pathogenesis. To explore the immunomodulatory potential of the studied therapies, anti-inflammatory (IL-4 and IL-10) and pro-inflammatory (IFN-γ) cytokines were quantified from J774A.1 macrophage cells (uninfected or infected with *T. cruzi*) exposed to the CC_{50} of the less cytotoxic microencapsulated (A16–160, A16–140, A8–140, ICS 95-GA, and ICS 95-M) and PEx.

In this study, levels of the anti-inflammatory IL-4 were below the detectable range in infected and uninfected cell controls (Fig. 5C). This cytokine remained untraceable when treated with BENZ. However, cocoa microencapsulates showed an anti-inflammatory potential in healthy cells without *T. cruzi* since a significant ascent of IL-4 was observed ($p<0.05$, compared to infected and uninfected controls). In this regard, ICS 95-M showed the highest levels of this cytokine (2.2 μg/mL, $p<0.0001$) (Fig. 5C).

About IL-10, all studied therapies reduced this interleukin in healthy macrophages, except ICS 95-M, which preserved the basal state (0.38 pg/mL) observed in the control cells (uninfected and untreated) (Fig. 5D). Conversely, *T. cruzi* infection induced a significant decline in IL-10 (0.28 pg/mL vs. 0.38 pg/mL for infected and uninfected cells, respectively; $p<0.0001$). The re-establishing of this interleukin levels (similar to uninfected control) was addressed by treatments of infected macrophages with BENZ and the microencapsulates A16–160 and ICS 95-M (0.38 pg/mL). These concentrations were significantly different from those observed for infected but untreated cells ($p<0.05$) (Fig. 5D).

According to these results, all cocoa extracts evidenced a significant anti-inflammatory potential by increasing the IL-4 in cells without *T. cruzi*-infection, ($p<0.0001$). ICS 95-M (from ICS-95 clone, freeze-dried and encapsulated in 16% maltodextrin) was also shown to re-establish IL-10 levels in these uninfected macrophages. Interestingly, this anti-inflammatory effect was selective for healthy cells and was not replicated in *T. cruzi* -infected macrophages. In contrast, the reference drug BENZ showed a significant deleterious effect on the IL-4 concentrations, in the same model (Fig. 5C and D).

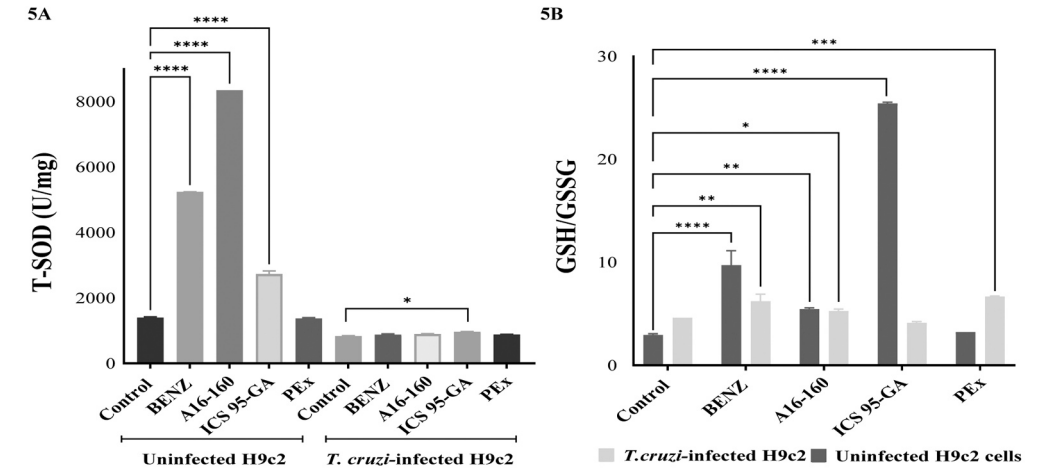
Related to IFN-γ (pro-inflammatory and antiparasitic cytokine), in healthy macrophages, the spray-dryer encapsulates treatments (as well as BENZ) induced lower values than the untreated control. Conversely, the two encapsulates obtained by freeze drying (ICS 95-M and ICS 95-GA) maintained them, with PEx as the only one that increased the concentration of this cytokine (0.62 pg/mL, $p<0.0001$) (Fig. 5E). On the other hand, macrophage infection by *T. cruzi* triggered a significant reduction (0.43 pg/mL vs. 0.25 pg/mL, $p<0.05$) and BENZ treatment was not able to reverse this situation. On the contrary, cocoa extracts caused an increase of this cytokine (compared to infected and untreated cells, $p<0.0001$); observing a complete restoration of the levels (relative to the uninfected control) in infected macrophages exposed to PEx, A8–140, and ICS 95-M (0.43 pg/mL) (Fig. 5E).

These results could explain the selective antiproliferative effect of the studied cocoa polyphenolic encapsulates on infected cells while observing cytoprotection on healthy macrophages and myocardioblast. On this point, the expression of IL-10 and IL-4 has been associated with preventing cardiac damage and improving cardiac function by triggering a possible immunomodulatory tolerance. Thus, IL-10 is considered a crucial regulatory mediator related to a favorable prognosis in chronic CHD, suggesting a protective effect for the Th1 response [6]. In this regard, anti-inflammatory cytokines such as interleukin IL-4 and IL-10, promote Th2 differentiation and limit Th1 response.

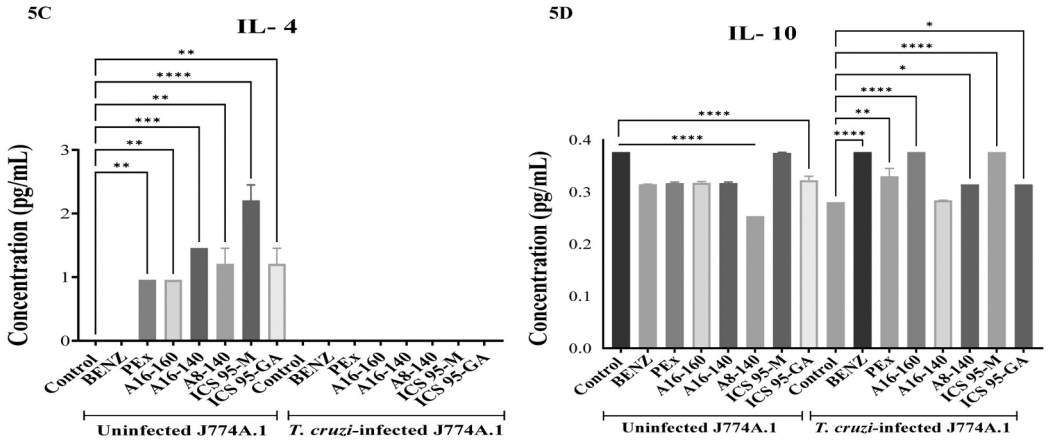
Consistently, in CCC models, the levels of this cytokine are generally declined, cooperating in generating a pro-inflammatory environment [9]. Additionally, a positive association between the expression of IL-10 and cardiac dysfunction (lower IL-10/worse cardiac function) has been described in models of chronic CHD [6]. These findings agree with studies showing that consuming flavonoid-rich chocolate or polyphenolic extracts from cocoa promotes the polarization of macrophage cells by favoring the phenotypic change M1 to M2. This effect was demonstrated by the decrease in the secretion of proinflammatory cytokines such as TNF-α, IL-6, IL-1β, and IL-12 and an improvement in anti-inflammatory ones such as IL-10 and IL-4, the latter being indispensable for the polarization process [21]. This polarization can permit *T. cruzi* persistence in cardiac tissue while keeping the inflammatory response under control, which could be crucial in preventing CCC progression [9].

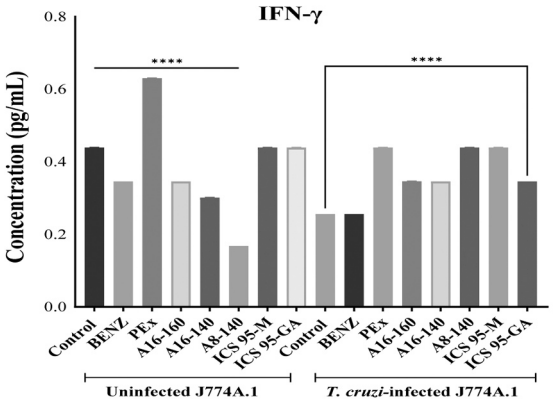
The extracts obtained from the different *T cacao* clones used in this study and identified using the analytical method UHPLC-ESI-Orbitrap-MS negative mode allowed the identification of epicatechin, as the main active principle. The estimated content of this flavonoid was found to be significantly associated with the trypanocidal action against *T. cruzi* amastigotes. No other compounds were found to be correlated with the *in vitro* bioactivities explored herein such as immunomodulatory (IL-4, IL-10, and IFN-γ) and antioxidant (T-SOD and GSH/GSSG levels) capability. However, other flavonoids with high pharmacological and antioxidant potential were identified including narangenin, rutin, luteolin, and quercetin.

Clinical trials and meta-analyses suggest that regular intake of cocoa-derived products rich in catechins contributes positively to the prevention of different diseases [56] and cardiovascular health [22,57]. Cocoa polyphenolic compounds are associated with cardioprotective processes



Treatment	T -SOD ± SD (U/mL)	p	T-GSH ± SD (µmol/10⁹)	p	% Reduced T-GSH	GSH ± SD (µmol/10⁹)	p	GSSG ± SD (µmol/10⁹)	p	GSH/GSSG
Uninfected H9c2 cells										
Control	1,393 ± 45.9	REF	45.3 ± 0.28	REF	59	26.8 ± 0.28	REF	9.2 ± 0.28	REF	2.9
BENZ	5,237 ± 11.3	<0.0001	56.3 ± 3.61	0.0055	83	46.5 ± 4.17	0.0014	4.8 ± 0.28	<0.0001	9.7
A16-160	8,341 ± 0	<0.0001	44.3 ± 0.35	NS	73	32.4 ± 0.5	0.0335	6 ± 0.07	0.0003	5.4
ICS 95-GA	2,722 ± 141	<0.0001	25.1 ± 0.14	0.0005	93	23.3 ± 0.14	NS	0.9 ± 0	<0.0001	25.4
PEx	1,368 ± 43.8	NS	68.1 ± 0.07	0.0003	61	41.9 ± 0.06	0.0038	13.1 ± 0.07	0.0001	3.2
***Trypanosoma cruzi* – infected H9c2 cells**										
Control	827 ± 27.1	REF	46.5 ± 0.71	REF	69	32.4 ± 0.59	REF	7.1 ± 0.07	REF	4.6
BENZ	868 ± 39.9	NS	31 ± 0.07	<0.0001	75	23.3 ± 0.68	0.0006	3.8 ± 0.28	0.0003	6.2
A16-160	888 ± 19	NS	57.1 ± 0.14	0.0002	72	41.3 ± 0.56	0.0007	7.9 ± 0.21	0.0453	5.2
ICS 95-GA	955 ± 19.1	0.0125	56 ± 0.07	0.0002	67	37.6 ± 0.35	0.0049	9.2 ± 0.21	0.0014	4.1
PEx	875 ± 17.3	NS	61.2 ± 1.20	<0.0001	76	47.1 ± 1.06	0.0001	7.1 ± 0.07	NS	6.7





Treatment	IL-4 ± SD (pg/mL)	p	IL-10 ± SD (pg/mL)	p	IFN-γ ± SD (pg/mL)	p
Uninfected J774A.1 cells						
Control	0.00 ± 0	REF	0.38 ± 0	REF	0.43 ± 0	REF
BENZ	0.00 ± 0	NS	0.31 ± 0	<0.0001	0.34 ± 0	<0.0001
PEx	0.95 ± 0	0.005	0.32 ± 0	<0.0001	0.62 ± 0	<0.0001
A16-160	0.95 ± 0	0.005	0.32 ± 0	<0.0001	0.34 ± 0	<0.0001
A16-140	1.45 ± 0	0.0005	0.31 ± 0	<0.0001	0.30 ± 0	<0.0001
A8-140	1.2 ± 0.3	0.0015	0.25 ± 0	<0.0001	0.16 ± 0	<0.0001
ICS 95-M	2.2 ± 0.3	<0.0001	0.37 ± 0	NS	0.43 ± 0	NS
ICS 95-GA	1.2 ± 0.3	0.0015	0.32 ± 0.01	<0.0001	0.43 ± 0	NS
***Trypanosoma cruzi* – infected J774A.1 cells**						
Control	0.00 ± 0	REF	0.28 ± 0	REF	0.25 ± 0	REF
BENZ	0.00 ± 0	-	0.38 ± 0	<0.0001	0.25 ± 0	NS
PEx	0.00 ± 0	-	0.33 ± 0	0.0018	0.43 ± 0	<0.0001
A16-160	0.00 ± 0	-	0.38 ± 0	<0.0001	0.34 ± 0	<0.0001
A16-140	0.00 ± 0	-	0.28 ± 0.04	NS	0.34 ± 0	<0.0001
A8-140	0.00 ± 0	-	0.31 ± 0,02	0.0147	0.43 ± 0	<0.0001
ICS 95-M	0.00 ± 0	-	0.38 ± 0	<0.0001	0.43 ± 0	<0.0001
ICS 95-GA	0.00 ± 0	-	0.31 ± 0	0.0147	0.34 ± 0	<0.0001

Fig. 5. Effects on antioxidant enzymatic systems and cytokines by microencapsulates of *T. cacao* L. in murine myocardioblasts (H9c2) and macrophages (J774A.1). A) Total superoxide dismutase (T-SOD); B) Reduced glutathione (GSH)/oxidized glutathione (GSSG) index; C) Interleukin (IL)−4; D) IL-10; E) Interferon (IFN)-γ; BENZ: benznidazole; PEx: crude pure extract. Control: untreated cells; cytokine tests performed with Cytotoxic Concentrations 50 (CC_{50})/Inhibitory Concentration 50 (IC_{50}), for uninfected/infected cells; antioxidant test performed with CC_{25}/IC_{50}, for uninfected/infected cells; A16-140 and A16-160: CCN51/Spray dried extracts encapsulated with gum arabic at 16% processed at 140°C or 160°C, respectively; ICS95-GA and ICS95-M: ICS-95/freeze-dried extracts encapsulated with gum arabic at 16%;* $p<0.05$; ** $p<0.01$; ** $p<0.001$; **** $p<0.0001$.

because they exhibit pro-inflammatory enzyme inhibitory activity [58]. Likewise, these polyphenol compounds in cocoa and dark chocolate may reduce the risk of cardiovascular events, lowering blood pressure, anti-atherosclerotic processes and positively activating endothelial function [59]. Thus, products containing high epicatechin content are more beneficial than when it is found in mixed matrices. It should be noted that the anti-inflammatory effects of this compound are associated with a reduction in heart attack [56,60].

Similar to our results, promissory anti-*T. cruzi* activity was been previously described for other natural polyphenols from sources other than cocoa, such as *Camellia sinensis* (green tea) [12], and phenolic extracts rich in catechins from *Morus nigra* (blackberry) [10]. The latter was also successful in reducing the inflammatory processes and increasing the levels of antioxidant enzymes [10]. Additionally, non-polyphenolic natural antioxidants such as curcumin (*Curcuma longa*) and resveratrol (red grapes) were shown to reduce the *T. cruzi*-parasite load in the heart and blood in experimental animal models of CHD, while improving cardiac and hepatic inflammation and survival rates [11]. Also, a complete reversion of cardiac damage was described in rats with CCC treated with essential oils of *L. alba* enriched in antioxidant terpenes, which also exhibited strong trypanocidal and immunomodulatory properties [9].

PEx exhibited the lowest cytotoxicity (CC_{50} values $>$ 7000 μg/mL) on J774A.1 and H9c2 cells, while preserving their morpho-physiological characteristics. Additionally, this compound presented the maximal trypanocidal activity against *T. cruzi*-amastigotes. However, among the studied cocoa compounds, this crude extract presented the lowest antioxidant (lowest activity of T-SOD and GSH/GSSH ratio, Fig. 5A and B) and anti-inflammatory potential (lowest IL-4/ IFN-γ ratio, Fig. 5C and E) in the health model of cardiac cells. Thus, a possible pro-oxidant response could have been triggered by the uncontrolled and rapid delivery of the polyphenols in the PEx-treated cells. This effect could be associated with the dual pro-oxidant/antioxidant behavior previously described for plant-derived polyphenols depending on their concentration, metal-reducing potential, chelating capacity, pH, and solubility [61]. Thus, the instability of the polyphenols in the pure extract could have led to its auto-oxidation, accounting for the ROS generation. Additionally, crude extracts are susceptible to important variations when entering living systems due to accelerated degradation (by pH, temperature, enzymes, or microbiota), biodistribution, photosensitivity, low hydrophobicity, and morphological barriers, among others [62]. These variations can be appropriately controlled by advanced delivery systems such as microencapsulation techniques, which can preserve the integrity and antioxidant capacity of the polyphenols by providing controlled and sustained delivery of high concentrations, solubility, stability, and easy transit between different biological barriers [49].

Among the novelties, we highlight that this study provides promising results for the development of bioproducts with technological innovation based on microencapsulation using cocoa active ingredients for cardioprotective purposes and an alternative treatment for individuals affected by Chagas disease. To continue with the development of this work and to have a traceability and quality of the extracts, it is recommended to perform chemical characterization of the same cocoa clones grown in a different edaphoclimatic zone than the one of this study, since the polyphenol profile may present variations. Likewise, it is of utmost importance to innovate with other alternative extraction techniques such as supercritical fluids, ultrasound, and microwave-assisted extraction, which allow extracting a greater concentration of polyphenols and being environmentally sustainable. Additionally, it is important to continue evaluating different encapsulating agents for spray-drying microencapsulation and to complement the results obtained herein by evaluating the microencapsulation of clones TSH-565, ICS-1, ICS-95, ICS-60, and IMC-67. To complement with kinetic studies of stability and protection of the powders obtained under different environmental conditions and to evaluate them in animal models.

4. Conclusion

The results of this work show a promising potential for microencapsulated extracts of *T. cacao*, rich in polyphenols (catechins and epicatechins), as platforms in the development of innovative individual or adjuvant therapies for the integral intervention of *T. cruzi* infection. The encapsulant gum arabic showed higher qualities as a cytoprotective agent, especially in preserving mitochondrial membrane potential and cellular architecture. Microencapsulated obtained by the spray-drying and coated in gum arabic represented the experimental model with the best solubility, stability, and handling characteristics. Their enhanced bioactivity was attributed to the higher content of epicatechins of clone CCN51 (clone of origin), which allows us to conclude that extract from this clone may be promising for the development of bioproducts such as nutraceuticals. Spray-dried microencapsulated extract A16–160 from clone CCN51 using encapsulation conditions of 16% gum arabic and 160 °C, and ICS 95-GA (obtained by freeze drying from ICS-95 clone encapsulated in 16% gum arabic) exhibited trypanocidal activity superior to that of BENZ, with antioxidant and immunomodulatory activities on healthy cells. This effect was attributed to the increase of IL-4 in uninfected cells; and to the rise of INF-γ and oxidized GSH in infected cells. To our knowledge, this is the first report of *T. cacao* microencapsulated rich in epicatechin with potential anti-*T. cruzi* activity. In addition, this type of polyphenol-rich microencapsulated with strong anti-inflammatory and antioxidant properties could also be applied in the clinical management of other chronic conditions typical of Western lifestyle and aging, such as atherosclerosis, neurodegenerative diseases, and cancer.

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CRediT authorship contribution statement

Angélica Sandoval-Aldana: Methodology, Investigation. **Wendy Quintero García:** Formal analysis, Data curation. **Laura Vargas-Munévar:** Methodology, Investigation, Formal analysis, Data curation. **Juan Borja-Fajardo:** Methodology, Investigation, Formal analysis, Data curation. **Elena Stashenko:** Investigation, Funding acquisition. **Liliana Torcoroma García:** Project administration, Methodology, Investigation. **Erika Moreno Moreno:** Methodology, Investigation, Formal analysis, Data curation. **Juan Camilo Henriquez:** Methodology. **Olimpo García:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2024.116307.

References

[1] K.M. Bonney, Chagas disease in the 21st Century: a public health success or an emerging threat ? Parasite *21* (2014) 11, https://doi.org/10.1051/parasite/2014012.

[2] K.C.F. Lidani, F.A. Andrade, L. Bavia, F.S. Damasceno, M.H. Beltrame, I.J. Messias-Reason, T.L. Sandri, Chagas disease: from discovery to a worldwide health problem, Front. Public Heal. *7* (2019) 166, https://doi.org/10.3389/fpubh.2019.00166.

[3] J.D. Whitman, Clinical microbiology, Clin. Microbiol. Newsl. *45* (2023) 141–149, https://doi.org/10.1016/j.clinmicnews.2023.09.001.

[4] B.Y. Lee, K.M. Bacon, M.E. Bottazzi, P.J. Hotez, Global economic burden of Chagas disease: a computational simulation model, Lancet Infect. Dis. *13* (2013) 342–348, https://doi.org/10.1016/S1473-3099(13)70002-1.

[5] K.M. Bonney, D.J. Luthringer, S.A. Kim, N.J. Garg, D.M. Engman, Pathology and pathogenesis of Chagas heart disease, Annu Rev. Pathol. *14* (2019) 421–447, https://doi.org/10.1146/annurev-pathol-020117-043711.

[6] A.C. Cristovão-Silva, M.C.A. Brelaz-de-Castro, M.Z. Hernandes, V.R.A. Pereira, Chagas disease: immunology of the disease at a glance, Cytokine Growth Factor Rev. *62* (2021) 15–22, https://doi.org/10.1016/j.cytogfr.2021.10.001.

[7] E. Maldonado, D.A. Rojas, F. Urbina, A. Solari, Review article the oxidative stress and chronic inflammatory process in Chagas disease: role of exosomes and contributing genetic factors, Oxid. Med. Cell. Longev. *2021* (2021), https://doi.org/10.1155/2021/4993452.

[8] F. Lascano, F.G. Bournissen, J. Altcheh, Review of pharmacological options for the treatment of Chagas disease, Br. J. Clin. Pharmacol. *88* (2022) 383–402, https://doi.org/10.1111/bcp.14700.

[9] D.X. Espinel-Mesa, C.I. González-Rugeles, J.C. Mantilla-Hernández, E.E. Stashenko, C.A. Villegas-Lanau, J.J. Quimbaya-Ramírez, L.T. García-Sánchez, Immunomodulation and antioxidant activities as possible trypanocidal and cardioprotective mechanisms of major terpenes from *Lippia alba* Essential oils in an experimental model of chronic Chagas disease, Antioxidants *10* (2021) 1851, https://doi.org/10.3390/antiox10111851.

[10] M.C. Montenote, V.Z. Wajsman, Y.T. Konno, P.C. Ferreira, R.M.G. Silva, A.L. S. Therezo, L.P.A. Martins, Antioxidant effect of Morus nigra on Chagas disease progression, Rev. Inst. Med. Trop. Sao Paulo 59 (2017) 1–11, https://doi.org/10.1590/S1678-9946201759073.

[11] R.D. Novaes, M.V.P. Sartini, J.P.F. Rodrigues, R.V. Gonçalves, E.C. Santos, R.L. M. Souza, I.S. Caldas, Curcumin enhances the anti- *Trypanosoma cruzi* activity of benznidazole-based chemotherapy in acute experimental Chagas disease, Antimicrob. Agents Chemother. *60* (2016) 3355–3364, https://doi.org/10.1128/AAC.00343-16.

[12] C. Paveto, M.C. Gu, I. Esteva, V. Martino, J. Coussio, M.M. Flawia, Anti-*Trypanosoma cruzi* activity of green tea (*Camellia sinensis*) catechins, Antimicrob. Agents Chemother. *48* (2004) 69–74, https://doi.org/10.1128/AAC.48.1.69.

[13] ICCO. International Cacao Organization. Available online: ⟨https://www.icco.org/aboutcacao/chocolate-industry.html⟩ (Accessed on 6 May 2023).

[14] FEDECACAO. Federación Nacional de Cacaoteros. El Cacaocultor es lo Primero. Economía Internacional. Available online: ⟨https://www.fedecacao.com.co/portal/index.php/es/2015-02-12-17-20-59/nacionales⟩ (Accessed on 6 October 2022).

[15] EFSA, Panel on dietetic products, nutrition and allergies (NDA). Scientific opinion on the substantiation of a health claim related to cocoa flavanols and maintenance of normal endothelium-dependent vasodilation pursuant to Article 13 (5) of Regulation (EC) No 1924/2006, EFSA J. *10* (2012) 2809, https://doi.org/10.2903/j.efsa.2012.2809.

[16] Y. Rodríguez-Carrasco, A. Gaspari, G. Graziani, A. Santini, A. Ritieni, Fast analysis of polyphenols and alkaloids in cocoa-based products by ultra-high performance liquid chromatography and Orbitrap high resolution mass spectrometry (UHPLC-Q-Orbitrap-MS/MS), Int. Food Res. J. *111* (2018) 229–236, https://doi.org/10.1016/j.foodres.2018.05.032.

[17] L. Dugo, G. Tripodo, L. Santi, C. Fanali, Cocoa polyphenols: chemistry, bioavailability and effects on cardiovascular performance, Curr. Med. Chem. *25* (2018) 4903–4917, https://doi.org/10.2174/0929867323666160919094339.

[18] D.L. Katz, K. Doughty, A. Ali, Cocoa and chocolate in human health and disease, Antioxid. Redox Signal *15* (2011) 2779–2811, https://doi.org/10.1089/ars.2010.3697.

[19] O. Yañez, M.I. Osorio, C. Areche, A. Vasquez-Espinal, J. Bravo, A. Sandoval-Aldana, W. Tiznado, *Theobroma cacao* L. compounds: theoretical study and molecular modeling as inhibitors of main SARS-CoV-2 protease, Biomed. Pharm. *140* (2021) 111764, https://doi.org/10.1016/j.biopha.2021.111764.

[20] J. Wollgast, E. Anklam, Review on polyphenols in Theobroma cacao: changes in composition during the manufacture of chocolate and methodology for identi ® cation and quanti ® cation, Int. Food Res. J. *33* (2000) 423–447, https://doi.org/10.1016/S0963-9969(00)00068-5.

[21] L. Dugo, M.G. Belluomo, C. Fanali, M. Russo, F. Cacciola, M. Maccarrone, A. M. Sardanelli, Effect of cocoa polyphenolic extract on macrophage polarization from proinflammatory M1 to anti-inflammatory M2 state, Oxid. Med. Cell Longe *2017* (2017), https://doi.org/10.1155/2017/6293740.

[22] Q.R. Araujo, Gattward De, J.N. Almoosawi, S. Conceição, G. Costa, P. Santana, P. A. De, G. Conceição, P. Costa, P. Alfredo, D.S. Dantas, et al., Cocoa and human health: from head to foot - a review, Crit. Rev. Food Sci. Nutr. *56* (2016) 1–12, https://doi.org/10.1080/10408398.2012.657921.

[23] S.A. Oyeleke, A.M. Ajayi, S. Umukoro, A.O. Aderibigbe, O.G. Ademowo, Anti-inflammatory activity of *Theobroma cacao* L. stem bark ethanol extract and its fractions in experimental models, J. Ethnopharmacol. *222* (2018) 239–248, https://doi.org/10.1016/j.jep.2018.04.050.

[24] N. Sari, Y. Katanasaka, H. Honda, Y. Miyazaki, Y. Sunagawa, M. Funamoto, T. Morimoto, Cacao bean polyphenols inhibit cardiac hypertrophy and systolic dysfunction in pressure overload-induced heart failure model mice, Planta Med. *86* (2020) 1304–1312, https://doi.org/10.1055/a-1191-7970.

[25] J.W. Li, X.Y. Wang, X. Zhang, L. Gao, L.F. Wang, X.H. Yin, -)-Epicatechin protects against myocardial ischemia-induced cardiac injury via activation of the PTEN/PI3K/AKT pathway, Mol. Med. Rep. *17* (2018) 8300–8308, https://doi.org/10.3892/mmr.2018.8870.

[26] H.D. Sesso, J.E. Manson, A.K. Aragaki, P.M. Rist, L.G. Johnson, G. Friedenberg, G. L. Anderson, Effect of cocoa flavanol supplementation for the prevention of cardiovascular disease events: the cocoa supplement and multivitamin outcomes study (COSMOS) randomized clinical trial, Am. J. Clin. Nutr. *115* (2022) 1490–1500, https://doi.org/10.1093/ajcn/nqac055.

[27] M. Darand, M. Hajizadeh-Oghaz, A. Hadi, M. Atefi, R. Amani, The effect of cocoa/dark chocolate consumption on lipid profile, glycemia, and blood pressure in diabetic patients: a meta-analysis of observational studies, Phytother. Res. *35* (2021) 5487–5501, https://doi.org/10.1002/ptr.7183.

[28] P. Vitaglione, R. Barone Lumaga, R. Ferracane, S. Sellitto, J.R. Morelló, J. Reguant Miranda, E. Shimoni, V. Fogliano, Human bioavailability of flavanols and phenolic acids from cocoa-nut creams enriched with free or microencapsulated cocoa polyphenols, Br. J. Nutr. *109* (2013) 1832–1843, https://doi.org/10.1017/S0007114512003881.

[29] L.F. Ballesteros, M.J. Ramirez, C.E. Orrego, J.A. Teixeira, S.I. Mussatto, Encapsulation of antioxidant phenolic compounds extracted from spent coffee grounds by freeze-drying and spray-drying using different coating materials, Food Chem. *237* (2017) 623–631, https://doi.org/10.1016/j.foodchem.2017.05.142.

[30] Z. Jiang, Z. Han, M. Zhu, X. Wan, L. Zhang, Effects of thermal processing on transformation of polyphenols and flavor quality, Curr. Opin. Food Sci. *51* (2023) 101014, https://doi.org/10.1016/j.cofs.2023.101014.

[31] V.L. Singleton, R. Orthofer, R.M. Lamuela-Raventós, Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent, Methods Enzym. *299* (1999) 152–178, https://doi.org/10.1016/S0076-6879(99)99017-1.

[32] I.F.F. Benzie, J.J. Strain, The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power", FRAP Assay. *239* (1996) 70–76, https://doi.org/10.1006/abio.1996.0292.

[33] W. Brand-Williams, M.E. Cuvelier, C.L.W.T. Berset, Use of a free radical method to evaluate antioxidant activity, LWT Food Sci. Techno *28* (1995) 25–30, https://doi.org/10.1016/S0023-6438(95)80008-5.

[34] B. Ou, M. Hampsch-woodill, R.L. Prior, Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe, J. Agric. Food Chem. *49* (2001) 4619–4626, https://doi.org/10.1021/jf010586o.

[35] W.L. Quintero, E.M. Moreno, S. Milena, L. Pinto, S.M. Sanabria, E. Stashenko, L. T. García, Immunomodulatory, trypanocide, and antioxidant properties of essential oil fractions of *Lippia alba* (Verbenaceae), BMC Complement Altern. Med. *21* (1) (2021) 16, https://doi.org/10.1186/s12906-021-03347-6.

[36] L.C. Carrillo, J. Londoño-Londoño, A. Gil, Comparison of polyphenol, methylxanthines and antioxidant activity in *Theobroma cacao* beans from different cocoa-growing areas in Colombia, FRIN *60* (2014) 273–280, https://doi.org/10.1016/j.foodres.2013.06.019.

[37] S. Kandasamy, R. Naveen, A review on the encapsulation of bioactive components using spray-drying and freeze-drying techniques, J. Food Process Eng. *45* (2022) 1–14, https://doi.org/10.1111/jfpe.14059.

[38] M.J. Payne, W.J. Hurst, K.B. Miller, C. Rank, D.A. Stuart, Impact of fermentation, drying, roasting, and dutch processing on epicatechin and catechin content of cacao beans and cocoa ingredients, J. Agric. Food Chem. 58 (2010) 10518–10527, https://doi.org/10.1021/jf102391q.

[39] G.E. Adamson, S.A. Lazarus, A.E. Mitchell, R.L. Prior, G. Cao, P.H. Jacobs, B. G. Kremers, J.F. Hammerstone, R.B. Rucker, K.A. Ritter, H.H. Schmitz, HPLC method for the quantification of procyanidins in cocoa and chocolate samples and correlation to total antioxidant capacity, J. Agric. Food Chem. *47* (1999) 4184–4188, https://doi.org/10.1021/jf990317m.

[40] S. Vertuani, E. Scalambra, T. Vittorio, A. Bino, A. Baldisserotto, S. Manfredini, Evaluation of antiradical activity of different cocoa and chocolate products: relation with lipid and protein composition, J. Med. Food *17* (2014) 512–516, https://doi.org/10.1089/jmf.2013.0110.

[41] V. Todorovic, I.R. Redovnikovic, Z. Todorovic, G. Jankovic, M. Dodevska, S. Sobajic, Polyphenols, methylxanthines, and antioxidant capacity of chocolates

produced in Serbia, J. Food Compost Anal. *41* (2015) 137–143, https://doi.org/10.1016/j.jfca.2015.01.018.
[42] S. Zapata-Bustamante, A. Tamayo-Tenorio, B. Alberto-Rojano, Efecto de la fermentación sobre la actividad antioxidante de diferentes clones de cacao colombiano, Rev. Cuba Plantas Med. *18* (2013) 391–404.
[43] L.J. Giraldo-López, Impact of fermentation and drying in polyphenol content and antioxidant capacity of cocoa variety CCN51, Rev. ION *29* (2016) 7–21, https://doi.org/10.18273/revion.v29n2-2016001.
[44] D.B. Haytowitz, S.U.S.D.A. Bhagwat, Database for the Oxygen Radical Absorbance Capacity (ORAC) of Selected, Foods, Release 2 *3* (2010) 10–48.
[45] A. Ricci, J.A. Arboleda, A. Versari, E. Chiarello, A. Bordoni, G.P. Parpinello, Microencapsulation of polyphenolic compounds recovered from red wine lees: process optimization and nutraceutical study, Food Bioprod. Process. *132* (2021) 1–12, https://doi.org/10.1016/j.fbp.2021.12.003.
[46] F. Felice, A. Fabiano, M. De-Leo, A.M. Piras, D. Beconcini, M.M. Cesare, A. Braca, Y. Zambito, R. Di Stefano, Antioxidant effect of cocoa by-product and cherry polyphenol extracts: a comparative study, Antioxidants *9* (2020) 1–14, https://doi.org/10.3390/antiox9020132.
[47] T.K. Kim, J.M. Hong, K.H. Kim, S.J. Han, I.C. Kim, H. Oh, J.H. Yim, Potential of ramalin and its derivatives for the treatment of alzheimer's disease, Molecules *26* (2021) 1–12, https://doi.org/10.3390/molecules26216445.
[48] M.J. TallonAuthorised EU health claim for cocoa flavanols. Elsevier Ltd.,2015, ISBN 9781782423829..
[49] Z. Fang, B. Bhandari, Spray drying, freeze drying and related processes for food ingredient and nutraceutical encapsulation, ESI (2012) 73–109, https://doi.org/10.1533/9780857095909.2.73.
[50] G. Ferreira, F. Matta, R. Augustus, D. Oliveira, Incorporation of spray dried and freeze dried blackberry particles in edible films: morphology, stability to pH, sterilization and biodegradation, Food Packag Shelf Life *20* (2019) 100313, https://doi.org/10.1016/j.fpsl.2019.100313.
[51] S. Krishnan, A.C. Kshirsagar, R.S. Singhal, The use of gum arabic and modified starch in the microencapsulation of a food flavoring agent, Carbohydr. Polym. *62* (2005) 309–315, https://doi.org/10.1016/j.carbpol.2005.03.020.
[52] R. Argüello-García, G.N. Quiñonez-Bastidas, Catechins as emerging and promising antiparasitic agents, Biomed. J. Sci. Technol. Res. *30* (2020) 23065–23071, https://doi.org/10.26717/BJSTR.2020.30.004895.
[53] V. Bolaños, A. Díaz-Martínez, J. Soto, M.A. Rodríguez, C. López-Camarillo, L. A. Marchat, E. Ramírez-Moreno, The flavonoid (−)-epicatechin affects cytoskeleton proteins and functions in *Entamoeba histolytica*. J. Proteom. *111* (2014) 74–85, https://doi.org/10.1016/j.jprot.2014.05.017.
[54] M.B.G. Dos Reis, L.C. Manjolin, C.D.C. Maquiaveli, O.A. Santos-Filho, E.R. Da Silva, Inhibition of *Leishmania* (Leishmania) *amazonensis* and rat arginases by green tea EGCG,(+)-catechin and (−)-epicatechin: a comparative structural analysis of enzyme-inhibitor interactions, PLoS One *8* (2013) e78387, https://doi.org/10.1371/journal.pone.0078387.
[55] J. Luo, H. Si, Z. Jia, D. Liu, Dietary anti-aging polyphenols and potential mechanisms, Antioxidants *10* (2021) 283, https://doi.org/10.3390/antiox10020283.
[56] I. Bernatova, Biological activities of (−)-epicatechin and (−)-epicatechin-containing foods: Focus on cardiovascular and neuropsychological health, Biotechnol. Adv. *36* (2018) 666–681, https://doi.org/10.1016/j.biotechadv.2018.01.009.
[57] J. Oracz, E. Nebesny, D. Zyzelewicz, G. Budryn, B. Luzak, Bioavailability and metabolism of selected cocoa bioactive compounds: a comprehensive review, Crit. Rev. Food Sci. Nutr. *60* (2020) 1947–1985, https://doi.org/10.1080/10408398.2019.1619160.
[58] F.A. Tomas-Barberán, E. Cienfuegos-Jovellanos, A. Marín, B. Muguerza, A. Gil-Izquierdo, B. Cerdá, J.C. Espín, A new process to develop a cocoa powder with higher flavonoid monomer content and enhanced bioavailability in healthy humans, J. Agric. Food Chem. *55* (2007) 3926–3935, https://doi.org/10.1021/jf070121j.
[59] A.C. Aprotosoaie, A. Miron, A. Trifan, V.S. Luca, I.I. Costache, The cardiovascular effects of cocoa polyphenols -an overview, Diseases *4* (2016) 39, https://doi.org/10.3390/diseases4040039.
[60] H. Schroeter, C.L. Keen, H.D. Sesso, J.E. Manson, J.R. Lupton, Is this the end of (-)-epicatechin, or not? New study highlights the complex challenges associated with research into the cardiovascular health benefits of bioactive food constituents, Am. J. Clin. Nutr. *102* (2015) 975–976, https://doi.org/10.3945/ajcn.115.117283.
[61] E.A. Decker, Phenolics: prooxidants or antioxidants? Nutr. Rev. *55* (1997) 396–398, https://doi.org/10.1111/j.1753-4887.1997.tb01580.x.
[62] M. Hu, B. Wu, Z. Liu, Bioavailability of polyphenols and flavonoids in the era of precision medicine, Mol. Pharm. *14* (2017) 2861–2863, https://doi.org/10.1021/acs.molpharmaceut.7b00545.