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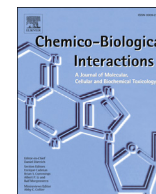

Oak kombucha protects against oxidative stress and inflammatory processes

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A B S T R A C T

Black tea infusion is the common substrate for preparing kombucha; however other sources such as oak leaves infusions can be used for the same purpose. Almost any white oak species have been used for medicinal applications by some ethnic groups in Mexico and could be also suitable for preparing kombucha analogues from oak (KAO). The objective of this research was to investigate the antioxidant activity and anti-inflammatory effects of KAO by examining its modulation ability on macrophage-derived TNF-alpha and IL-6. Herbal infusions from oak and black tea were fermented by kombucha consortium during seven days at 28 °C. Chemical composition was determined by LC-ESI-MS/MS. The antioxidant activity of samples against oxidative damage caused by H_2O_2 in monocytes activated (macrophages) was explored. Additionally, it was determined the anti-inflammatory activity using lipopolysaccharide (LPS) - stimulated macrophages; in particular, the nitric oxide (NO), TNF-alpha, and IL-6 production was assessed. Levels of pro-inflammatory cytokines IL-6 and TNF-alpha were significantly reduced by the sample treatment. Likewise, NO production was lower in treatment with kombucha and KAO compared with LPS-stimulated macrophages. Fermented beverages of oak effectively down-regulated the production of NO, while pro-inflammatory cytokines (TNF-alpha and IL-6) in macrophages were stimulated with LPS. Additionally, phytochemical compounds present in KAO decrease oxidative stress.

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1. Introduction

Some fermented foods have transcended their sources to become everyday products in more than one continent; fermentations involved in these foods are of enormous complexity, and their study has provided us a wealth of biotechnology knowledge. An attractive bioprocess consists on the degradation of glucose and fructose through the fermentation action of a bacterial and yeast consortium called Kombucha [6]. This Kombucha is a fermented beverage that has been traditionally consumed in China for over 2200 years. This ancient beverage is composed of two portions: a floating biofilm of cellulose and the sour liquid broth [4]. Several positive effects have been reported, including gastro protective effect of the culture broth and probiotic potential of the Kombucha microbiome [1,13]. In particular, in the culture broth the main metabolites identified are gluconic and glucuronic acids, glycerol, phenolic acids and caffeine; some are associated with beneficial effects on health. The two main classes of involved polyphenols are flavonoids and phenolic acids. Their chemical and structural modifications are due to biotransformation and metabolism by the kombucha consortium action, and have not been taken into account in previous studies of kombucha analogues obtained from other sources. The biotransformation of flavonoids has been a topic of research due to the interest in explaining the correlation between the beneficial properties of flavonoids and the structures of the active compounds. In Kombucha obtained from black tea, the

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epigallocatechin-3-gallate biotransformation to epigallocatechin, epicatechin-3-gallate and epicatechin by enzymes excreted by kombucha microbiome has been demonstrated [9].

Concordantly, the development of functional beverages fermented from new sources that not only mitigates the consumer's thirst, but also contains phytochemicals that provide protective effects to health, is currently considered an innovative area with high value-added potential. Fermentation with the consortium Kombucha of infusions made from waste defoliation of oak has proven to be a viable alternative to obtain beverages rich in bioactive metabolites and increased bioavailability, which gives the advantage of consuming more bio-effective products [25]. Thus, using fermentation technology for the development of analogues of Kombucha may help to propose healthier food alternatives to the population.

Most biological activities *in vitro* have been tested using the aglycone forms of polyphenols. However in nature, flavonoids are conjugated with sugars, which can affect the antioxidant properties of compounds. Furthermore, recent studies have demonstrated the limited bioavailability of most polyphenols and the role of conjugated species such as glycosides or glucuronides in the absorption and circulating forms in the body. Although deglycosylation is likely to occur either in pre- or post-absorption, metabolism *in vivo* of these compounds may lead to neo-conjugation of one or more hydroxyl groups with sulphate and glucuronic acids [14]. The antioxidant properties of polyphenols are generally associated with the presence of *ortho* phenolic groups and the nature and position of these substitutions affect subsequent biological activities, possibly reducing or suppressing activities detected in the aglycone forms.

It is difficult to distinguish the effect of oak leaves infusion composition over the development of kombucha consortium, because chemical compounds present in infusion have demonstrated antimicrobial activity [22]. Until now, there are few reports describing the use of oak as a substrate for Kombucha analogues [25]. Moreover, there are no reports in the literature describing the metabolic profiles produced by the consortium kombucha after consumption of phenolic compounds. Since polyphenols present in this source are important from their antioxidant, antihypertensive and anti-inflammatory properties, it is therefore necessary to study the content and fate of bioactive polyphenols in fermented beverages.

Polyphenols, as a group of secondary metabolites broadly distributed in natural products, are in general considered as health promoters by their antioxidant activity [16]. Some polyphenols from oak leaves infusions include catechin, quercetin, kaempferol, naringin, naringenin and ellagic acid [19] among other compounds. In *Quercus* species, have also been reported hydrolysable tannins as vescalagin and castalagin [18] as well as bioactive proanthocyanidins [21]. These sources have proven to exhibit antimicrobial activity against some pathogens [22], anticarcinogenic and antioxidant potential [15]. However, infusions obtained from oak cause astringent or bitter taste [20]. Most polyphenolic compounds exhibit astringency and to be consumed with pleasure is necessary to mask their taste [12]. Therefore, the biological activity of polyphenols is complex, suggesting further investigations on these metabolites and their properties [23].

In this study, some white species such as *Quercus resinosa*, *Q. arizonica* and *Q. convallata* (formerly classified as *Q. obtusata*) were explored as potential sources for obtaining Kombucha analogues from oaks (KAO), evaluating changes on their phenolic compounds, sugar and organic acids by LC-ESI-MS/MS, and their antioxidant capacity and anti-inflammatory potential in a cellular model of macrophages.

2. Materials and methods

2.1. Reagent and biological materials

Starter Kombucha consortium (Healthy, Natural Life, Tlaquepaque, Jal., Mexico). THP-1 human monocytic cells were obtained from American Type Culture Collection (ATCC). Catechin, epicatechin, gallocatechin, gallocatechin gallate, epicatechin-gallate, rutin, myricetin, kaempferol, kaempferol 3-O-glycoside, quercetin, quercetin glucuronide, naringin, phloridzin, gallic acid, 3,4-dihydroxybenzoic acid, chlorogenic acid, 4-hydroxybenzoic acid, 4-O-caffeoylquinic acid, caffeic acid, 2,4,6, trihydroxybenzaldehyde, coumaric acid, ferulic acid, 3,4-dicaffeoylquinic acid, 4,5-dicaffeoylquinic acid, salicylic acid, succinic acid, acetic acid, glucuronic acid, gluconic acid, fructose, glucose, sucrose, RPMI 1640 medium, fetal bovine serum, L-glutamine, sodium pyruvate, penicillin, streptomycin, phorbol-12-mirystate-13-acetate,3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, lipopolysaccharide, commercial ELISA kit (TNF-alpha and IL-6), hydrogen peroxide, 2,3-diaminophthalene, dichlorofluoroscein-diacetate were obtained from Sigma Chemical (St. Louis, MO, USA), acetonitrile LC-MS grade (J.Baker).

Quercus resinosa leaves were obtained from trees located at 9.2–9.4 km in Mezquital-Charcos Road in Southern Durango, Mexico, *Q. arizonica* leaves and *Q. convallata* leaves were collected from trees located at 53–54 km in El Tecuán – Regocijo road in Durango, Mexico. The leaves were air dried in the shade at 25 °C followed by milling to a particle size of 0.7–100 mm.

2.2. Preparation of herb infusions

Infusions were prepared by adding 1 g of ground material to 100 mL of water and allowed to stand for 10 min at 80 °C, followed by centrifugation at 4500 rpm for 10 min and then filtered using a 0.5 mm pore size filter.

2.3. Started cultures and fermentation

The started Kombucha was maintained in sweetened (sucrose 10%) black tea at 25 °C. The freshly cultured Kombucha was used for further subcultures of fresh fermentation batches.

The fermentation conditions were previously established with *Quercus resinosa* species according to [25]: fermentation time (7 days), sugar concentration (10%), starter culture (10%), inoculum of consortium (2.5%) and temperature (25 °C). The study was extended to fermenting infusions of *Quercus* spp. from white species (*Q. resinosa*, *Q. arizonica*, and *Q. convallata*).

2.4. Chemical characterization

Detection and quantification of major compounds was achieved using electrospray ionization/tandem spectrometry in multiple reaction-monitoring mode (MRM) to follow transitions of molecules into their specific fragmentation ions. Calibration curves for each compound were created by plotting standard concentrations (x-axis) and peak area ratios (y-axis) using linear regression and the concentration of 28 reference compounds in the sample calculated according to the slope from their standard curves.

2.5. Sugar, gluconic and glucuronic acid content in fermented beverages

The LC system consisted of a sample manager (5 °C) and a binary solvent manager coupled with a tandem Xevo TQ-S triple quadrupole mass spectrometer (Waters Corp., Milford, MA, USA). The

column used to determine fructose, glucose and sucrose was an Acquity UPLC BEH Amide, 100 mm × 2.1 × 1.7 μm (Waters Corp., Milford, MA, USA) operated at 50 °C. The elution profile used two solvents, acetonitrile/water with 0.1% NH_4OH (80/20) (A), and acetonitrile/water with 0.1% NH_4OH (30/70) (B): gradient from 0 to 60%B in 5 min, 60%B by 1min, then from 60 to 5%B in 0.5min, reset and equilibrated for 5 min. The flow rate was 0.25 mL/min. The control and data processing were performed using Masslinx (Waters Corp., Milford, MA, USA) software.

2.6. Phenolic content analysis

Sample analysis was carried out with an Acquity UPLC system (Waters Corp., Milford, MA, USA) coupled with a tandem Xevo TQ-S triple quadrupole mass spectrometer (Waters Corp., Milford, MA, USA). The LC system consisted of a sample manager (10 °C) and a binary solvent manager. Phenolic compounds were determined with an Acquity UPLC BEH C8 100 mm × 2.1 × 1.7 μm column (Waters Corp., Milford, MA, USA) operated at 30 °C according to [7] with slight modifications. The elution profile used to quantify flavan-3-ols, flavonols, and phenolic acids included two solvents, acidified water with 7.5 mM formic acid (A) and acetonitrile LC-MS (B): initial 98%A in B; 0–2 min, 68%A in B; 2–3.8min, 55%A in B; 3.8–4.5min 45%A in B; 4.5–6.0min 5%A in B (linear gradient) for column washing; and subsequently 6.0–9.5 min, 98%A in B for column stabilization. MRM data were collected from 0 to 13.5 min. Negative ionization mode was used for MS assays. ESI conditions were as follows: capillary voltage 2.85 kV; desolvation temperature 500 °C; source temperature, 150 °C; desolvation and cone gas, 794 and 151 L/h, respectively, and collision gas, 0.14 mL/min. Rutin (20 ng/μL) was used to monitor the stability of the ionization efficiency of the mass spectrometer and a mixture of different phenolic compounds (20 ng/μL) was used to monitor retention time and *m/z* values. For quantification of several flavonoids and phenolic acids, a calibration curve was done. The UPLC and tandem Xevo TQ-S triple quadrupole mass spectrometer control and data processing were performed using MassLinx (Waters Corp., Milford, MA, USA) software.

2.7. Antioxidant and anti-inflammatory effect

2.7.1. Cell culture

THP-1 human monocytic cells were routinely grown in RPMI 1640 medium with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate and a mixture of antibiotics. Incubation was carried out at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Monocyte differentiation to macrophages was carried out according to [2]. Briefly, 10 ng/mL of PMA (phorbol-12-mirystate-13-acetate) for 48 h; after treatment, cells were thoroughly washed with PBS and RPMI 1640 supplemented medium was added. Cells were incubated for 72 h and used for oxidative stress and anti-inflammatory assays.

2.7.2. Cell viability

Macrophages viability was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-ditetrazolium bromide (MTT) method [26]. Unless otherwise stated, cells were seeded into 96 well plates at a density of 3.5×10^4 cells per well in 0.2 mL RPMI 1640 medium with 10% FBS and antibiotics. After the experimental treatments, macrophages were thoroughly washed with PBS, then 0.2 mL of free red-phenol medium containing 1 mg/mL of MTT were added and cells incubated for 4 h. The MTT reduced by the viable cells to formazan was solubilized with 0.2 mL of DMSO and absorbance was measured at a test wavelength of 570 nm and a reference wavelength of 690 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VE, USA).

2.8. Experimental treatments

Several concentrations from the lyophilized Kombucha (*C. sinensis*) and KAO were prepared in RPMI 1640 medium. The culture media were evaluated in terms of morphological change, pH, osmolality, viscosity, and precipitation of components in absence of serum in order to avoid possible cytotoxic effects due to changes in these physicochemical factors over the cellular microenvironment as described by Ref. [15]. Only the concentrations that did not change the culture media conditions were selected for further experiments. Time of treatment (4 h) was established for exploration of the anti-inflammatory effect. Finally, a dose range of 2–200 μg/mL was established to build viability dose response curves and to determine concentrations to test a cytotoxic effect in macrophages. Dimethyl sulfoxide in RPMI medium (0.02%) was used as negative control.

2.9. Suppression of fermented beverages on TNF-alpha and IL-6 release in LPS-stimulated macrophages

Macrophages were incubated with KAO formulated with *Q. resinosa*, *Q. arizonica*, *Q. convallata* and kombucha of *Camellia sinensis* used as control, at a concentration of 20 μg/mL for 4 h in RPMI medium. After this treatment, the macrophages were stimulated with LPS (100 ng/mL, dissolved in 10 mM hepes in PBS) for 6 h. Supernatants were collected for analysis of cytokines (TNF-alpha and IL-6) using a commercial ELISA kit.

2.10. Fluorimetric determination of nitrite with 2,3-diaminonaphthalene (DAN)

Determination of nitric oxide was assessed by the method proposed by Ref. [17]. Briefly, supernatants (100 μL) were incubated with 30 μL DAN (25 μg/mL in 0.62 N HCl) at room temperature in the dark for 10min. The mixture was adjusted to pH 11.5 with NaOH 2.8 N. The fluorescence was measured with a fluorimeter (Jasco FP-8300, Easton, MD, USA) at an excitation of 365 nm and emission wavelengths of 450 nm. Nitrite concentrations were calculated from standard curves as described above.

2.11. Measurement of reactive oxygen species induced by H_2O_2

Cells were plated at 3.4×10^4 and differentiated as described above in 96-well plates. Cells were incubated for 4 h with treatments at several concentrations. Later, cells were loaded with dichlorofluoroscein-diacetate (4 μM) and incubated for 15 min at 37 °C. Then cells were washed with PBS and added to reaction with H_2O_2 (100 μM) for 1 h. Fluorescence was then measured by a fluorimeter (Jasco FP-8300, Easton, MD, USA) set to 485 nm excitation and 530 nm emission wavelengths at 37 °C.

2.12. Statistical analysis

All results were expressed as the mean ± standard deviation. Data were analyzed by one-way ANOVA and differences among treatments were determined by comparison of means using Tukey and Dunnett tests using Statistica v7.2, (StatSoft, Tulsa, OK, USA). The level of statistical significance was considered at $p < 0.05$.

3. Results and discussion

KAO obtained from *Q. resinosa* metabolized intensively sucrose with fast production of kombucha floating biofilm. In a first stage of

the fermentation process of oak infusions with kombucha consortium, sucrose biogenesis was observed at 24 h with an increase of disaccharide concentration of 58.4% in *Q. resinosa*, 23.2% in *Q. arizonica* and 27.4% in *Q. convallata* (Fig. 1). Since sucrose concentration in *C. sinensis* decreased at 24 h by 16.3% followed by a disaccharide biogenesis in the next 24 h of 25.0%, we are speculating that this biogenesis may be attributed to the symbiotic action of *Gluconoacetobacter xylinus* with other microorganisms present in the consortium; however, this was not demonstrated in this research. Further, it is well established that sucrose is a source of carbon in the system, which is first hydrolyzed releasing fructose and glucose. In this context, the main residual monosaccharide in fermented beverages in all treatments was glucose, monomer necessary for the synthesis of the cellulose biofilm. It is well known that Kombucha metabolizes in first instance fructose that serves as an energy source of carbon. Residual glucose is mainly the substrate for synthesis of the final product; this is oxidized to gluconic acid by the action of catalase present in the system and to glucuronic acid (GlcUA). The beneficial effects attributed to kombucha tea have been associated to these metabolites and their polyphenolic content. Most documented investigations indicate that as a result of the fermentation process with consortium kombucha, high concentrations of gluconic acid are produced. In fact, this metabolite had applications in pharmaceutical industry due to its properties as a chelating agent for calcium and iron, so that several researchers have evaluated different sources to obtain it. A relevant characteristic of gluconic acid is that it imparts a refreshing acidic taste to wines and fruit juices. In addition, gluconic acid is spontaneously isomerized to glucono.

δ-lactone (metabolite not determined), which has antiseptic properties with an initially sweet taste, becoming slightly acidic. Recently, it has been explored the selective conversion of glucose from strawberry purée into gluconic acid, with conversion efficiencies of 20 g/L [3], which are similar to those obtained in the KAO explored in this research (Fig. 2a), even though in this study was not observed a decrease of glucose in any system assay.

GlcUA present in fermented beverages such as kombucha is a relevant metabolite considered as a modified form of glucose in which the alcohol group at the 6th position is replaced with a carboxylic group. This metabolite participates in detoxification processes in the human organism when is conjugated to xenobiotics. In the present research, GlcUA was detected at low concentrations (Fig. 2b) with a similar response than those documented in other studies at fermentation times near to seven days [10]. In this context, it must be considered that the fermentation conditions related to sugar concentration and fermentation times influences the production of these metabolites. Particularly, for fermentation with oak infusions, the production of GlcUA reached levels of 48.7–60.5 mg/L after seven days of fermentation at 28 °C.

A special feature detected in the fermented beverages, was the glucuronidation of phytochemicals of phenolic nature mainly in the first 24 h of fermentation process. This phenomenon was followed with a quercetin-3-O-β-glucuronide standard (Fig. 2c), finding a higher concentration of this metabolite in the KAO formulated with *Q. resinosa* and *Q. arizonica* infusions. Furthermore, the production of myricetin glucuronide was analyzed, identifying its presence according to its fragmentation pattern (Fig. 2d), determining that beverages formulated with *Q. arizonica* and *Q. convallata* have a higher abundance on this metabolite. It is relevant to highlight that the production of these metabolites is carried out during the first 24 h of the fermentation process at pH 3.34–3.40 (Fig. 1). The above makes us to postulate the presence of UDP-glucuronosyltransferase (UGT) related compounds in the Kombucha fermentation system.

Researchers have carried out studies that have led to a new direction in the use of fermentation systems when applying the kombucha consortium on herbal teas from traditional medicine. So far, it is well known that tea leaves are rich in polyphenols. These compounds have antioxidant properties and great abundance in natural products. Its preventive role as regards for several pathologies is associated with reducing oxidative stress. A source rich in phenolic compounds is the bark and leaves of *Quercus* commonly known as oak. Oak leaf infusions contain high amounts of polyphenols, and are used by local ethnic groups in México as a refreshing drink since old times [20]. Table 1 show the profile and content of phenolic compounds present in fermented beverages obtained from oak infusions, and compared against Kombucha prepared with black tea as principal source of polyphenols. Polyphenol compounds have been extensively studied for their antioxidant and cardio-protective effects, and as a consequence in the modulation of oxidative stress [5]. The antioxidant activity of flavonoids, in particular, is attributed to the presence of ortho hydroxyl groups, due to their ability for scavenging and avoiding the formation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) by delocalization of electrons [24]. demonstrated that the antioxidant activity of quercetin 3-O-β-glucuronide towards ROS in mouse fibroblasts is more effective when quercetin is in its aglycone form due to the instability of this compound in the culture medium.

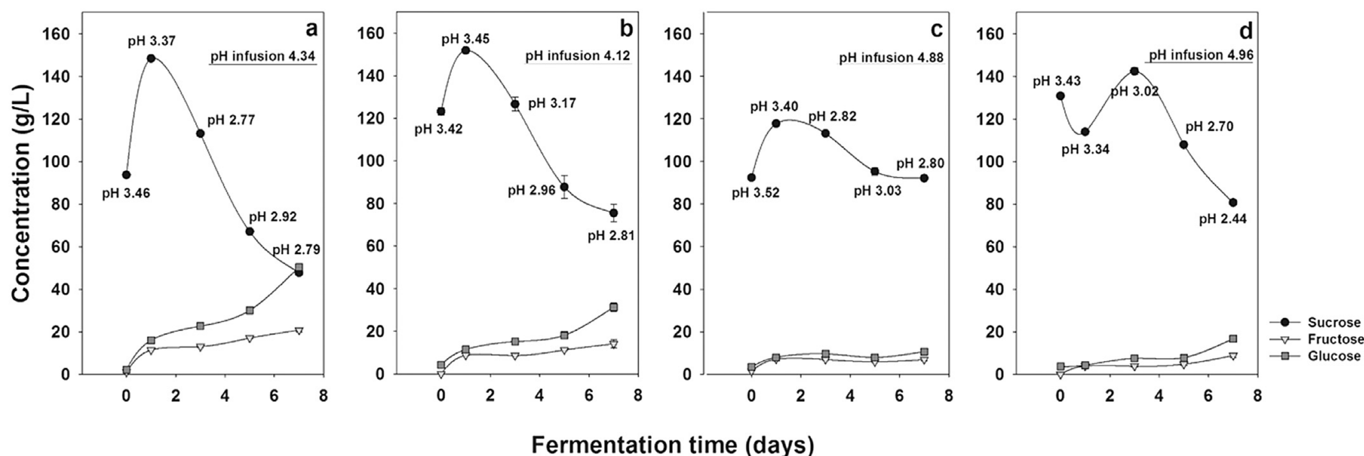

Fig. 1. Effect of the kombucha consortium in herbal infusions of white oak species and black tea, on catabolism and biogenesis of carbohydrates. **a)** *Q. resinosa*, **b)** *Q. arizonica*, **c)** *Q. convallata* and d) *C. sinensis*. Data are expressed as the mean value ± standard deviation of triplicate samples.

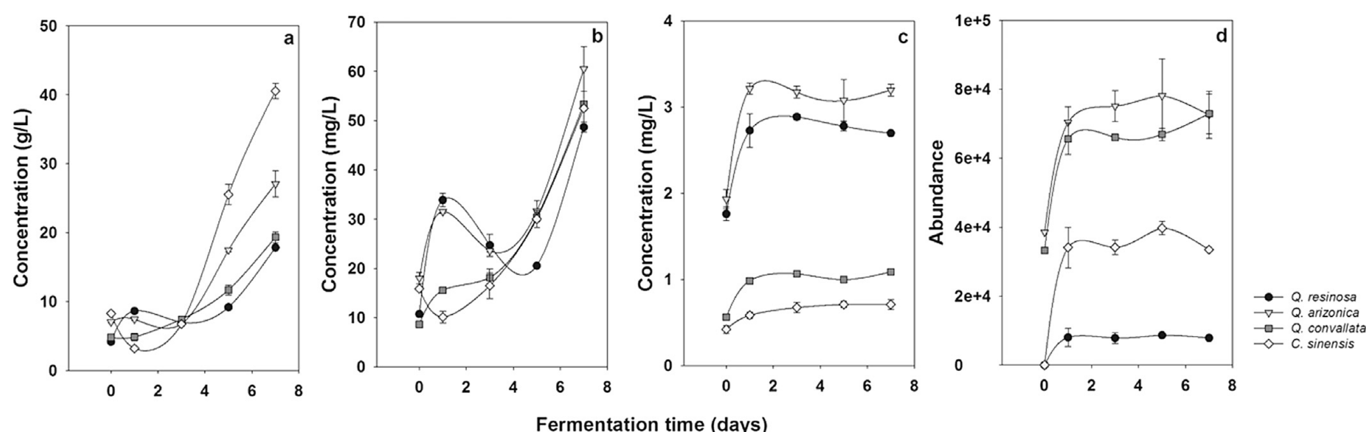

Fig. 2. Effect of the kombucha consortium in herbal infusions of white oak species (*Quercus* spp) and black tea (*Camelia sinensis*), on production of metabolites. **a)** Gluconic acid, **b)** Glucuronic acid, **c)** Quercetin 3-o-β-glucuronide and d) Mirycetin-glucuronide. Data are expressed as the mean value ± standard deviation of triplicate samples.

Based on the chemical characterization performed in this study (Table 1), we have detected compounds that may be recommended for their prophylactic effects, which are harmless to humans and possess nutraceutical properties. It should be noted that is not necessarily to find a direct relationship between the consumption of fermented herbal beverages and their subsequent biological activities in a specific cell and tissue model [27]. In this research were have detected concentrations of catechin and epicatechin in black tea Kombucha of 8.453 ± 0.64 and 142.62 ± 0.91 mg/mL, respectively. The concentration of catechin in KAOs were for *Q. resinosa* 33.241 ± 0.96 mg/L, *Q. arizonica* 9.22 ± 0.35 mg/L and *Q. convallata* 10.94 ± 0.88 mg/L. Fermented beverage of *Q. convallata* has a gallocatechin content (34.46 ± 1.58 mg/L) similar to the black tea Kombucha (34.64 ± 0.59 mg/L). Phenolic acids detected in oak's beverages were gallic acid, 3,4 dihydroxy-benzoic acid, 2,4,6-tri-hydroxybenzaldehyde, salicylic acid, chlorogenic acid, 4-O-caffeoylquinic acid, caffeic acid, coumaric acid, ferulic acid, 4,5 di-caffeoylquinic acid and 3,4 di-caffeoylquinic acid, some of these compounds in concentrations lower than those detected in *C. sinensis*, a material used in this research as a reference product.

Considering the impact of oxidative stress in atherosclerotic processes, attempts have been made to study new products rich in phenolic compounds that reduce the damage induced by ROS and RNS. However, there are factors influencing the bioavailability of the antioxidant compounds, including the composition of the beverage, its absorption and metabolism. Thus the presence of glucuronide compounds in experimental samples allows an approximation to the response that would be obtained in an *in vivo* system. Furthermore, chemical methods used for determining the antioxidant capacity *in vitro* do not differentiate the action of single antioxidant compounds but the collective effect of a mixture of active compounds, so limiting the investigation of the precise biological mechanisms to the synergism effect of their action [27]. Therefore, the selection of a suitable method for characterizing the biological potential of beverages obtained from natural products depends on the composition and stability of products and other

Table 1
LC-ESI-MS/MS profile and content of phenolic compounds present in fermented beverages from white oak leaves (*Quercus* spp) and black tea (*Camelia sinensis*).

No.	Compound	t_R (min)	$[M-H]^-$ *m/z*	MS/MS ions	Concentration (mg/L)			
					Q. resinosa	*Q. arizonica*	*Q. convallata*	*C. sinensis*
1	Gallic acid	1.67	169	125, 79	16.640 ± 0.83^a	19.566 ± 1.33^b	19.195 ± 1.09^b	54.396 ± 5.02^c
2	Gallocatechin	2.56	305	164, 125	8.485 ± 1.33^a	8.561 ± 0.65^a	34.466 ± 1.58^b	34.640 ± 0.59^b
3	3,4 di-hydroxy-benzoic acid	3.20	153	109,81	1.866 ± 0.04^a	2.126 ± 0.083^b	0.284 ± 0.036^c	0.587 ± 0.00^d
4	Catechin	4.46	289	203, 109	33.841 ± 0.96^a	9.225 ± 0.35^b	10.948 ± 0.88^c	8.453 ± 0.64^b
5	Chlorogenic acid	4.47	353	191, 135	0.149 ± 0.01^a	0.302 ± 0.014^b	0.355 ± 0.029^b	0.539 ± 0.07^c
6	4-hydroxy-benzoic acid	4.57	137	93	0.000 ± 0.00	0.000 ± 0.00	0.000 ± 0.00	0.355 ± 0.05
7	4-O-caffeoylquinic acid	4.65	353	191, 173	0.013 ± 0.001^a	0.015 ± 0.00^a	0.019 ± 0.00^a	0.102 ± 0.00^b
8	Caffeic acid	5.03	179	135, 107	0.175 ± 0.08^a	0.156 ± 0.04^a	0.000 ± 0.00^b	16.213 ± 1.18^c
9	Epicatechin	5.18	289	162, 125	14.417 ± 0.36^a	8.141 ± 0.11^b	10.037 ± 0.09^c	142.62 ± 0.91^d
10	Gallocatechin gallate	5.32	457	305, 169, 125	4.553 ± 0.00^a	5.248 ± 0.26^b	6.000 ± 0.02^c	58.799 ± 1.23^d
11	Rutin	6.30	609	300, 271	4.341 ± 0.06^a	2.786 ± 0.31^b	0.646 ± 0.07^c	4.245 ± 0.27^a
12	2,4,6-tri-hydroxy-benzaldehyde	6.37	153	107, 93	0.000 ± 0.00^a	0.015 ± 0.00^b	0.000 ± 0.00^a	0.067 ± 0.01^c
13	Coumaric acid	6.38	163	119, 93	0.252 ± 0.00^a	0.182 ± 0.02^{ac}	0.054 ± 0.00^b	0.174 ± 0.02^c
14	Quercetin glucuronide	6.61	477	301, 151	2.697 ± 0.02^a	3.196 ± 0.06b	1.086 ± 0.02^c	0.708 ± 0.05^d
15	Epicatechin gallate	6.73	441	289,169	1.584 ± 0.05^a	1.196 ± 0.03^b	1.135 ± 0.04^b	18.365 ± 1.07^c
16	Ferulic acid	6.75	193	178, 149	0.052 ± 0.00^a	0.168 ± 0.00^b	0.026 ± 0.00^c	0.000 ± 0.00^a
17	4,5 di-caffeoylquinic acid	7.28	515	353, 191	0.005 ± 0.00^a	0.004 ± 0.00^a	0.001 ± 0.00^a	0.008 ± 0.00^a
18	Kaempferol 3-O-glycoside	7.34	447	285	3.739 ± 0.16^a	0.000 ± 0.00^b	2.221 ± 0.25^c	0.748 ± 0.00^d
19	Naringin	7.65	579	271, 151	0.008 ± 0.00^a	0.003 ± 0.00^b	0.004 ± 0.00^b	0.000 ± 0.00^a
20	3,4 di-caffeoylquinic acid	7.78	515	353, 179	0.012 ± 0.00	0.003 ± 0.00	0.000 ± 0.00	0.005 ± 0.00
21	Myricetin	7.86	317	179, 151	0.000 ± 0.00^a	0.000 ± 0.00^a	0.000 ± 0.00^a	0.184 ± 0.00^b
22	Salicylic acid	7.93	137	93	0.000 ± 0.00^a	0.078 ± 0.02^b	0.080 ± 0.00^b	0.000 ± 0.00^a
23	Phloridzin	8.17	471	435, 271	0.742 ± 0.059^a	0.681 ± 0.05^a	0.449 ± 0.02^b	0.038 ± 0.01^c

Data are expressed as the mean value ± standard deviation of triplicate samples; different literals in lines represent significant difference between samples (Tukey, $p < 0.05$).

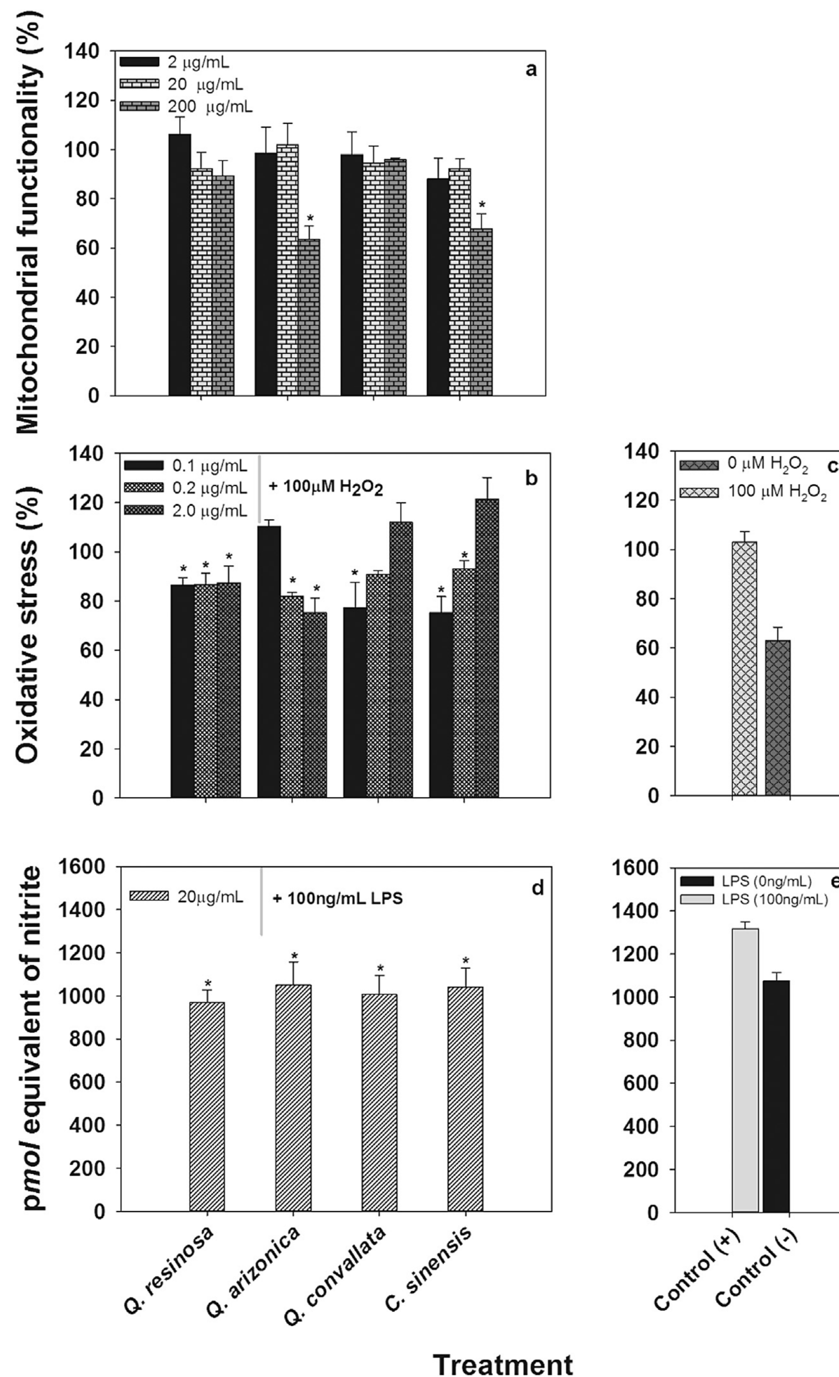

Fig. 3. Effect of fermented beverages on cell viability and oxidative stress: a) mitochondrial functionality, b) inhibition of oxidative stress induced with H2O2 and treated with extracts, c) treatment with H2O2 without extracts, d) inhibition of reactive nitrogen species induced with LPS (100 ng/mL) and treated with extracts, e) treatment with LPS without extracts. Data are expressed as the mean value ± standard deviation of triplicate samples; different literals in lines represent significant difference versus control group (Dunnett, $P < 0.001$).

factors, including the synergistic effect in the antioxidant activity or their possible pro-oxidant activity under specific conditions, among other mechanisms of action as was observed for *C. sinensis* and *Q. convallata,* whose have shown a pro-oxidative doses-response.

To assess effect of KAO on the cell viability of macrophages, the MTT reduction by mitochondrial dehydrogenases was determined after treatment with different concentrations of lyophilized extracts (2–200 μg/mL), which did not affect the cellular environment (osmolality, viscosity, pH, precipitation in the culture medium). No representative inhibition of MTT reduction was observed after addition of 20 μg/mL or lower concentrations (Fig. 3a), allowing to establish this concentration for anti-inflammatory assays and to determine its effect in inhibiting the formation of RNS.

In this context, the cell culture system, using macrophages differentiated from THP-1 cells, was active with lipopolysaccharide (LPS) at 100 ng/mL leading to production of high levels of nitric

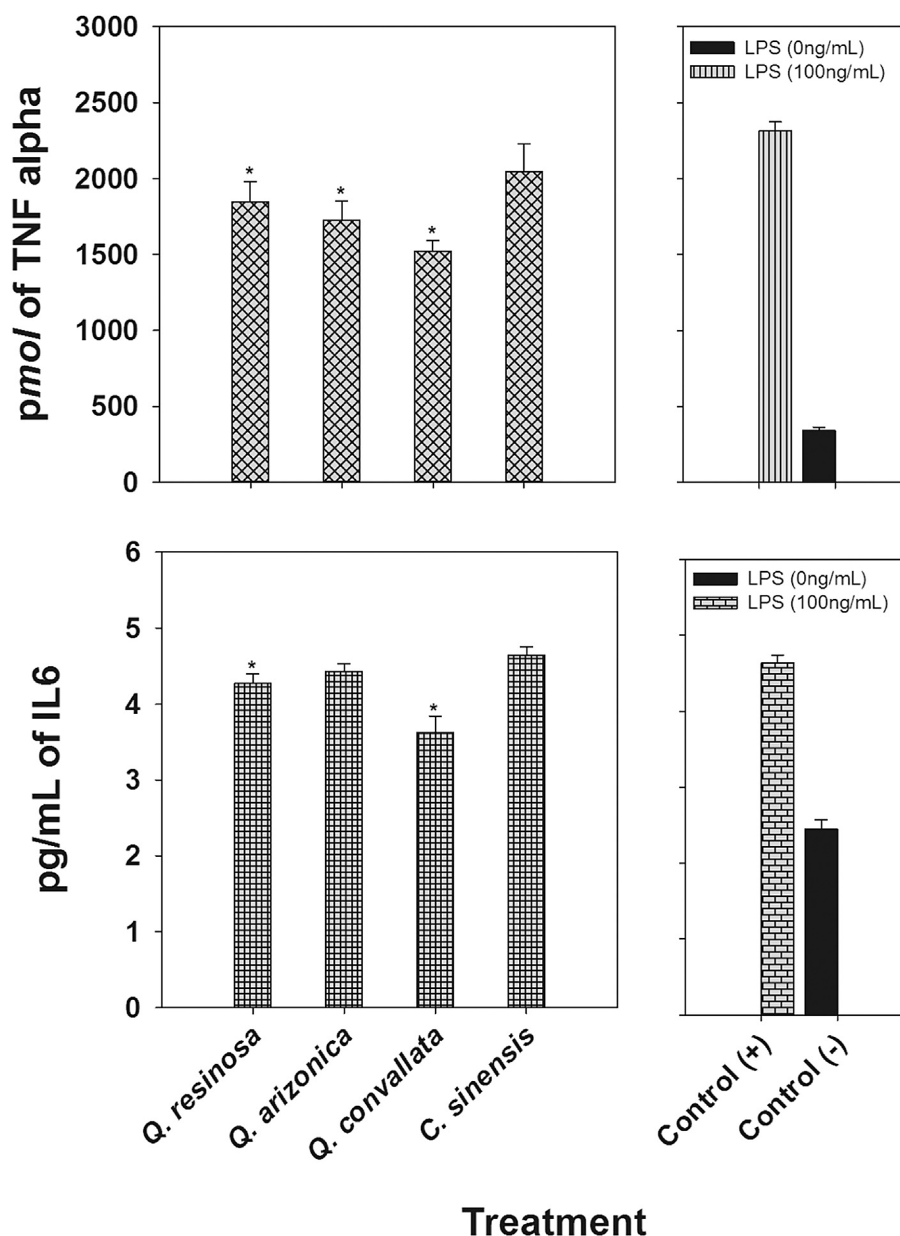

Fig. 4. Effect of fermented beverages on inflammatory targets: **a)** TNF-alpha expression levels (pre-treatment for 4 h with extract more 6 h in co-treatment with LPS (100 ng/mL)), **b)** TNF-alpha expression levels with LPS without extracts, **c)** IL-6 expression levels (pre-treatment for 4 h with extract more 6 h in co-treatment with LPS (100 ng/mL)), **d)** IL-6 expression levels with LPS without extracts. Data are expressed as the mean value ± standard deviation of triplicate samples; different literals in lines represent significant difference versus control group (Dunnett, $P < 0.001$).

oxide (NO). In macrophages, NO is synthesized by iNOS to destroy cells that have been phagocytosed, and plays an important role in the host immune response, where high production of NO is involved in acute inflammatory conditions. NO prevents adhesion and infiltration of leukocytes, the expression of pro-inflammatory molecules such as NF-kB, adhesion molecules (ICAM-1 and VCAM-1) and oxidation of LDL. However, an increase in iNOS may lead to a higher generation of reactive oxygen and nitrogen species, aggravating atherogenesis; on the other hand, the absence of iNOS also induces the formation of the lesion. NO is highly unstable and in the presence of the superoxide anion may react forming peroxynitrite, a toxic molecule that cause oxidative damage in proteins and lipids. According to our results, fermented beverages treatments maintain the nitrite concentration at similar levels to those obtained with the controls without induction of LPS damage (Fig. 3). This response is associated also with the ability of phytochemicals to compete for O_2 and inhibit the oxidation of nitric oxide.

As previously mentioned, polyphenolic compounds have been extensively studied for their antioxidant effects, and as a consequence in their capacity for modulation of oxidative stress [5]. The inhibitory effects of phenolic compounds such as quercetin glucuronide, present in fermented beverages (Fig. 2c), are associated witdh activity/expression of cyclooxygenase-2 and iNOS. The accumulation of evidences suggests that oxidative stress alters endothelial functions, including modulation of the vasomotor tone, promoting the oxidation of NO by superoxide anions and other reactive oxygen species. Endothelial dysfunction is the mayor event

in the development of atherosclerosis and heart attack, despite the normal or increased production of NO by the endothelium. As a consequence of oxidative stress, the vascular bioavailability of NO as the most important vasodilator is reduced.

Consequently, when the body is overwhelmed with free radicals, RNS or ROS involved in the physiological regulation of different organs and cellular activities, virtually all biological structures from the genetic material to proteins, carbohydrates and lipids become a vulnerable target, leading cell to its death. In this context, it is useful to know the antioxidant capacity of fermented beverages, since the current therapeutic approaches recommend the intake of products rich in compounds with nutraceutical potential. The study of KAO showed greater efficacy in their ability to scavenging ROS compared with positive control treated with 100 μM of H_2O_2 (Fig. 3b). To determine this response, the concentration of treatments was reduced by one order of magnitude, given the strong pro-oxidative effect that was shown at concentrations above 2 μg/mL.

To investigate the anti-inflammatory activity of extracts, ELISA kit was used to quantify in supernatants produced the pro-inflammatory cytokines TNF-alpha and IL6. These cytokines are prime targets that play a critical role in acute and chronic inflammatory diseases, in such a way that natural products capable of suppressing them are considered as anti-inflammatory therapeutic agents. Certain phytochemicals are known to possess immune suppressive activity, including phenolic compounds. In our experimental samples when tested at 20 μg/mL, compared with activated monocytes (macrophages) and stimulated with LPS, they significantly suppressed the TNF-alpha levels induced by LPS(Fig. 4a and b). Manny studies have demonstrated that chemical compounds present in fermented beverages of *Quercus* possess anti-inflammatory activity by suppressing TNF-alpha and IL-6. For example, naringin present in oaks significantly suppressed the LPS-induced production of NO, and the expression of inflammatory gene products such as iNOS, TNF-alpha and IL-6 [11]. Additionally, (+)-catechin inhibits the production of nitric oxide and TNF-alpha in LPS-stimulated macrophages [8]. An important compound present in oak fermented products is quercetin glucuronide. When evaluating the response associated with secreted cytokine TNF-alpha in treatments using oak's beverages, a high correlation was found ($R = 0.8208$). Likewise, at the same concentration, the treatments of *Q. resinosa* and *Q. convallata* also significantly suppressed LPS-induced IL-6 levels (Fig. 4c) with a higher correlation value ($R = 0.999$). The present research demonstrates an immune modulatory activity of *Quercus* species by suppression of the proinflammatory cytokines TNF-alpha and IL-6 by LPS-stimulated macrophages.

4. Conclusion

In this work we have demonstrated that kombucha analogues from oak show good antioxidant properties attributed particularly to their phenolic composition. The major effect was detected in their ability to suppress LPS – induced production of NO, TNF-alpha and IL-6, showing an important anti-inflammatory activity.

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References

[1] D. Banerjee, S. Hassarajani, B. Maity, G. Narayan, S. Bandyopadhyay, S. Chattopadhyay, Comparative healing property of kombucha tea and black tea against indomethacin-induced gastric ulceration in mice: possible mechanism of action, Food & Funct. 1 (3) (2010) 284–293, http://dx.doi.org/10.1039/C0FO00025F.

[2] H.S. Berg-Rolness, Optimized Differentiation of THP-1 Cells into Macrophages for the Study of TLR4 Signaling and Trafficking Events, Norwegian University of Science and Technology, 2014.

[3] A.M. Cañete-Rodríguez, I.M. Santos-Dueñas, M.J. Torija-Martínez, A. Mas, J.E. Jiménez-Hornero, I. García-García, Preparation of a pure inoculum of acetic bacteria for the selective conversión of glucose in strawberry purée into gluconic acid, Food Bioprod. Process. 96 (2015) 35–42, http://dx.doi.org/10.1016/j.fbp.2015.06.005.

[4] C. Chen, B.Y. Liu, Changes in major components of tea fungus metabolites during prolonged fermentation, J. Appl. Microbiol. 89 (2000) 834–839, http://dx.doi.org/10.1046/j.1365-2672.2000.01188.x.

[5] S.J. Duffy, J.F. Keaney Jr., M. Holbrook, N. Gokce, P.L. Swerdloff, B. Frei, J.A. Vita, Short-and long-term black tea consumption reverses endothelial dysfunction in patients with coronary artery disease, Circulation 104 (2) (2001) 151–156, http://dx.doi.org/10.1161/01.CIR.104.2.151.

[6] V. Franco, J. Perín, V. Mantovani, H. Goicoechea, Monitoring substrate and products in a bioprocess with FTIR spectroscopy coupled to artificial neural networks enhanced with a genetic-algorithm-based method for wavelength selection, Talanta 68 (6) (2006) 1005–1012, http://dx.doi.org/10.1016/j.talanta.2005.07.003.

[7] J. Gruz, O. Novák, M. Strnad, Rapid analysis of phenolic acids in beverages by UPLC-MS/MS, Food Chem. 111 (2008) 789–794, http://dx.doi.org/10.1016/j.foodchem.2008.05.014.

[8] C. Guruvayoorappan, G. Kuttan, (+)-Catechin inhibits tumour angiogenesis and regulates the production of nitric oxide and TNF-α in LPS-stimulated macrophages, Innate Immun. 14 (3) (2008) 160–174, http://dx.doi.org/10.1177/1753425908093295.

[9] R. Jayabalan, S. Marimuthu, K. Swaminathan, Changes in content of organic acids and tea polyphenols during kombucha tea fermentation, Food Chem. 102 (2007) 392–398, http://dx.doi.org/10.1016/j.foodchem.2006.05.032.

[10] R. Jayabalan, R.V. Malbasa, E.S. Loncar, J.S. Bitas, M. Sathishkumar, A review on kombucha tea-microbiology, composition, fermentation, beneficial effects, toxicity, and tea fungus, Compr. Rev. Food Sci. Food Saf. 13 (4) (2014) 538–550, http://dx.doi.org/10.1111/1541-4337.12073.

[11] S-i Kanno, A. Shouji, A. Tomizawa, T. Hiura, Y. Osanai, M. Ujibe, Y. Obara, N. Nakahata, M. Ishikawa, Inhibitory effect on naringin on lipopolysaccharide (LPS)-induced endotoxin shock in mice and nitric oxide production in RAW 264.7 macrophages, Life Sci. 78 (2006) 673–678, http://dx.doi.org/10.1016/j.lfs.2005.04.051.

[12] S.L. Kosaraju, D. Labbert, M. Emin, I. Konczak, L. Lundin, Delivering polyphenols for healthy ageing, Nutr. Diet. 65 (Suppl. 3) (2008) S48–S52, http://dx.doi.org/10.1111/j.1747-0080.2008.00261.x.

[13] N.O. Kozyrovska, O.M. Reva, V.B. Goginyan, J.-P. de Vea, Kombucha microbiome as a probiotic: a view from the perspective of post-genomics and synthetic ecology, Biopolym. Cell. 28 (2) (2012) 103–113.

[14] C. Marvarin, R. Azerad, Microbial glucuronidation of polyphenols, J. Mol. Catal. B Enzym. 73 (2011) 43–72, http://dx.doi.org/10.1016/j.molcatb.2011.07.015.

[15] M.R. Moreno-Jiménez, F. Trujillo-Esquivel, M.A. Gallegos-Corona, R. Reynoso-Camacho, R.F. González-Laredo, J.A. Gallegos-Infante, N.E. Rocha-Guzmán, M. Ramos-Gómez, Antioxidant, anti-inflammatory and anticarcinogenic activities of edible red oak (*Quercus* spp.) infusions in rat colon carcinogenesis induced by 1,2-dimethylhydrazine, Food Chem. Toxicol. 80 (2015) 144–153, http://dx.doi.org/10.1016/j.fct.2015.03.011.

[16] H. Mukhtar, N. Ahmad, Tea polyphenols: prevention of cancer and optimizing health, Am. J. Clin. Nutr. 71 (6) (2000) 1698s–1702s.

[17] A.K. Nussler, M. Glanemann, A. Schirmeier, L. Liu, N.C. Nüssler, Fluorometric measurement of nitrite/nitrate by 2, 3-diaminonaphthalene, Nat. Protoc. 1 (5) (2006) 2223–2226, http://dx.doi.org/10.1038/nprot.2006.341.

[18] S. Peng, A. Scalbert, B. Monties, Insoluble ellagitannins in *Castanea sativa* and *Quercus petraea* woods, Phytochemistry 30 (1991) 775–778, http://dx.doi.org/10.1016/0031-9422(91)85250-4.

[19] M.J. Rivas-Arreola, N.E. Rocha-Guzmán, J.A. Gallegos-Infante, R.G. González-Laredo, M. Rosales-Castro, J. Bacon, R. Cao (Tsao), A. Proulx, M.P. Intriago-Ortega, Antioxidant activity of oak leaves infusions against free radicals and their cardioprotective potential, Pak. J. Biol. Sci. 13 (11) (2010) 537–545, http://dx.doi.org/10.3923/pjbs.2010.537.545.

[20] N.E. Rocha-Guzmán, J.R. Medina-Medrano, J.A. Gallegos-Infante, R.F. González-Laredo, M. Ramos-Gómez, R. Reynoso-Camacho, H. Guzmán-Maldonado, S.M. González-Herrera, Chemical evaluation, antioxidant capacity and consumer acceptance of several oak infusions, J. Food Sci. 77 (2) (2012) 162–166, http://dx.doi.org/10.1111/j.1750-3841.2011.02524.x.

[21] M. Rosales-Castro, R.F. González-Laredo, N.E. Rocha-Guzmán, J.A. Gallegos-Infante, M.J. Rivas-Arreola, J.J. Karchesy, Antioxidant activity of fractions from *Quercus sideroxyla* bark and identification of proanthocyanidins by HPLC-DAD and HPLC-MS, Holzforschung 66 (2012) 577–584, http://dx.doi.org/10.1515/hf-2011-0157.

[22] J.A. Sánchez-Burgos, M.V. Ramírez-Mares, M.M. Larrosa, J.A. Gallegos-Infante,

R.F. González-Laredo, L. Medina-Torres, N.E. Rocha-Guzmán, Antioxidant, antimicrobial, antitopoisomerase and gastroprotective effect of herbal infusions from four *Quercus* species, Ind. Crop. Prod. 42 (2013) 57–62, http://dx.doi.org/10.1016/j.indcrop.2012.05.017.

[23] A. Scalbert, G. Williamson, Dietary intake and bioavailability of polyphenols, J. Nutr. 130 (8S Suppl) (2000) 2073S–2085S.

[24] M. Shirai, J.H. Moon, T. Tsushida, J. Terao, Inhibitory effect of a quercetin metabolite, quercetin 3-O-β-D-glucuronide, on lipid peroxidation in liposomal membranes, J. Agric. Food Chem. 49 (11) (2001) 5602–5608, http://dx.doi.org/10.1021/jf010713g.

[25] B.D. Vazquez-Cabral, S.M. González-Herrera, R.F. González-Laredo, M.R. Moreno-Jiménez, I.T. Córdova-Moreno, J.A. Gallegos-Infante, N.E. Rocha-Guzmán, Chemical and sensory evaluation of a functional beverage obtained from infusions of oak leaves (*Quercus resinosa*) inoculated with Kombucha consortium at different processing conditions, Nutrafoods 13 (2014) 169–178, http://dx.doi.org/10.1007/s13749-014-0035-0.

[26] J. Van Meerloo, G.J. Kaspers, J. Cloos, Cell sensitivity assays: the MTT assay, in: Cancer Cell Culture. Methods and Protocols, vol. 731, Methods Mol. Biol. Humana Press, 2011, pp. 237–245, http://dx.doi.org/10.1007/978-1-61779-080-5_20.

[27] F. Virgili, M. Marino, Regulation of cellular signals from nutritional molecules: a specific role for phytochemicals, beyond antioxidant activity, Free Radic. Biol. Med. 45 (9) (2008) 1205–1216, http://dx.doi.org/10.1016/j.freeradbiomed.2008.08.001.