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Phenolic composition of selected herbal infusions and their anti-inflammatory effect on a colonic model *in vitro* in HT-29 cells

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Abstract: Some herbal infusions used in folk medicine in Mexico to treat gastrointestinal disorders were evaluated. Antioxidant activity and phenolic compounds were analyzed on the lyophilized aqueous crude extracts (LACE) of arnica (*Aster gymnocephalus*), chamomile (*Chamaemelum nobile*), cumin (*Cominum cyminum*), desert resurrection plant (DRP) (*Selaginella lepidophylla*), laurel (*Listea glaucescens*), marjoram (*Origanum majorana*), mint (*Mentha spicata*), salvilla (*Buddleia scordioides*) and yerbaniz (*Tagetes lucida*). Total phenolic content ranged from 8.0 to 70.7 µg GAE/mg for DRP and laurel respectively. Major phenolic compounds were identified by gas chromatography–mass spectrometry and high-performance liquid chromatography. The IC_{50} determined by the degradation of the deoxy-D-ribose ranged from 2,452.53 to 5,097.11 µg/mL. The cytoprotective effect of the LACE alone and on indomethacin-induced oxidative stress in HT-29 cells was tested. The tetrazolium dye MTT assay was performed in concentrations of 0.125–10 mg/mL allowing choosing the lowest concentration for this experimentation. Inflammation markers were measured by

Elda Herrera-Carrera

ABOUT THE AUTHORS

The authors are part of the "Functional Foods and Nutraceuticals Academic Group", a research team at the Technological Institute of Durango (TNM-ITD). Their endeavor is to educate specialists as graduate students and to perform research on the subject. Their search focuses on scientific and technological options to use and convert local natural resources into bioactive and functional products, which may contribute to health and diet solutions for the general population. Among their current investigation topics are local plants with known biological activity such as Quercus and guava leaves, which also are tested as kombucha analog products. They also pursue the utilization of natural polymers from mucilages of "nopal" (*Opuntia* spp.) and Aloe vera plants. Another matter of interest is the development of nanomaterials and organogels to encapsulate natural principles and nutraceuticals.

PUBLIC INTEREST STATEMENT

Stomach pain and gastrointestinal inflammation are common conditions in people with bad diets or eating disorders. Another cause is the unsupervised consumption of non-steroidal anti-inflammatory drugs (NSAIDs). Traditionally, herbal teas, infusions, and decoctions of many plants have been used to treat such complications with no exact knowledge on what phytochemicals and metabolic mechanisms are involved. Arnica, chamomile, cumin, desert resurrection plant, laurel, marjoram, mint, salvilla, and yerbaniz are typical examples of herb infusions consumed in Mexico to alleviate these ailments. In this paper, the herbal infusions were freeze dried and analyzed for polyphenols as bioactive antioxidant compounds present in plants that might be responsible for the protective role of these beverages. Extracts were also tested in a colonic cell model and inflammation markers determined, comparing against a positive control from a NSAID like indomethacin. It follows that salvilla, chamomile, and laurel have shown promising and explainable anti-inflammatory effects.

Western blotting. None of the extracts inhibited COX-1 by themselves; however, it was observed that extracts have a modulation effect over COX-2, TNFα, NFκB, and IL-8. By the decrease in the expression of pro-inflammatory cytokines, it follows that salvilla, chamomile, and laurel show promising anti-inflammatory effects.

Subjects: Beverages; Food Analysis; Nutraceuticals & Functional Foods

Keywords: herbal infusion; polyphenols; anti-inflammatory activity; HT-29

1. Introduction

The use of natural products for the prevention and treatment of different pathologies is continuously expanding throughout the world. Plant derivate dietary agents consist of a wide variety of biologically active compounds and many of them have been used as traditional medicines for thousands of years (Aggarwal & Shishodia, 2006). Herbal infusions are aromatic beverages prepared by pouring hot or boiling water over dry plants (Figueroa-Pérez, Rocha-Guzmán, Mercado-Silva, Loarca-Piña, & Reynoso-Camacho, 2014). Tea and herbal infusions contribute to the consumption of phenolic compounds in our diet and have been studied for their phenolic content, antioxidant activity and phenolic profile (Shahidi, 2000). In Mexico, some herbs are traditionally used in folk medicine as infusions (tea) to treat stomach pain and gastrointestinal inflammation problems (González Elizondo, López Enriquez, González Elizondo, & Tena Flores, 2002). In recent years, there has been an increasing interest in bioactive compounds from herbal plants due to their health benefits. Phenolic compounds have attracted great attention due to both their large distribution among dietary components and the variety of biological activities that they display, so natural products are recovering space and importance in the pharmaceutical industry as inspiring sources of new potentially bioactive molecules (Rocha-Guzmán et al., 2009).

Inflammation is the primary response to infection or injury and is a complex pathophysiological process that can be measured by the expression of various markers such as NF-κB, TNFα, and COX-2 (Yu-Jin, Eun-Ju, Haeng-Ran, & Kyung-A, 2013). Inflammation causes an increase in oxidative stress that is produced by reactive oxygen species (ROS), and which is associated with many pathological disorders (Halliwell & Gutteridge, 1989). Oxygen-free radicals are continuously formed as intermediates of enzymatic reactions during normal cellular functions and some might be involved in growth, regulation, and intracellular signaling (Rahman, Biswas, & Kirkham, 2006). In living organisms, the levels of free radicals and other reactive species are controlled by a complex web of antioxidant defenses, which minimizes oxidative damage to biomolecules. In human disease, this oxidant–antioxidant balance is tilted in favor of the reactive species, so that oxidative damage levels increase (Halliwell, 2001).

Plant polyphenols are well recognized for their antioxidant activities. As antioxidants, polyphenols may protect cell constituents against oxidative damage and therefore, limit the risk of various degenerative diseases associated with oxidative stress. Antioxidants are capable of stabilizing, or deactivating free radicals from attacking cells and biological targets (Atoui, Mansouri, Boskou, & Kefalas, 2005). Effects of polyphenols such as anti-inflammatory, anti-tumor, and antiatherogenic could not be explained solely on the basis of their antioxidant properties. Investigations on the mechanisms of action of these molecules have proven the fact that polyphenols may not exert their effects as free radical scavengers, but also modulate cellular signaling processes during inflammation or may themselves serve as signaling agents (Aggarwal & Shishodia, 2006).

Consumption of non-steroidal anti-inflammatory drugs (NSAIDs) is one of the causes of gastrointestinal disorders worldwide. The NSAIDs, which include indomethacin, increase ROS generation (Giardina, Boulares, & Inan, 1999). To protect the intestine from mucosal injury induced by NSAIDs is an important issue in the field of gastroenterology. Drugs developed for this purpose like the prostaglandin E1 (PGE_1) derivate, and anti-acids including proton pump inhibitors cause side effects,

therefore some alternatives could be ROS-quenching agents (Omatsu et al., 2010). Phytochemicals such as polyphenols are effective free radical scavengers and metal chelators. There might be a reason why herbal infusions have been one common remedy used since aforetime. Hundreds of polyphenols with antioxidant activity are potential contributors to the antioxidant mechanisms present in humans and animals. Although these compounds are excellent candidates to explain the health benefits of diets rich in fruits and vegetables, there is still not enough information on food composition data, bioavailability, interaction with other food components, and their biological effects. It is possible that dietary polyphenols continuously participate in the regulation of cellular function independently of its antioxidant properties (Rahman et al., 2006).

Many plants are traditionally used by the native population in Mexico to help in the treatment of gastrointestinal ailments. The plants tried for the present study were: arnica (*Aster gymnocephalus*), chamomile (*Chamaemelum nobile*), cumin (*Cominum cyminum*), desert resurrection plant (DRP) (*Selaginella lepidophylla*), laurel (*Listea glaucescens*), marjoram (*Origanum majorana*), mint (*Mentha spicata*), salvilla (*Buddleia scordioides*), and yerbaniz (*Tagetes lucida*). Lyophilized infusions from these plants were analyzed for their beneficial properties that might be attributed to their phenolic compounds. Total phenolic content (TPC), identification of phenolic compounds, and hydroxyl radical scavenging capacity were evaluated attributing the anti-inflammatory effect to these compounds and their antioxidant capacity related to an anti-inflammatory effect.

The aim of this study was to prove the anti-inflammatory effect of the lyophilized aqueous crude extracts (LACE) by themselves and on indomethacin-induced oxidative stress and inflammation by following some inflammatory markers in an *in vitro* model since these plant infusions are already used by the population with the empirical knowledge that they help to cure intestinal disorders.

2. Materials and methods

2.1. Plant materials

Salvilla plant (*B. scordioides*) was collected in Colonia Minerva, Durango, Mexico, and fully identified by Socorro Gonzalez-Elizondo at CIIDIR-IPN Herbarium with voucher number 42018. Salvilla was dried at room temperature before using it. The other eight plants were purchased at a local market in Durango, Mexico. These samples are typically collected in wild form, but were botanically identified at the same Herbarium, although it was not possible to file a formal voucher specimen.

2.2. Reagents

Folin Ciocalteu reactive (2N), deoxy-D-ribose, thiobarbituric acid (TBA), trichloroacetic acid (TCA), 3-(4,5-di-methylthiazol-2-yl)-2,5,-dephenyl-2 tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), indomethacin, Roswell Park Memorial Institute 1640 (RPMI-1640) medium, trypsin, penicillin, and streptomycin were obtained from Sigma Chemical (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from bio-West (Kansas City, MO, USA). Primary monoclonal antibodies for COX-1 (sc-1752), Cox-2 (sc-1745), TNFα (sc-1350, NF-κB p65 (sc-372), IL-8 (sc-7922), GAPDH (sc-25778); secondary antibodies mouse anti-goat IgG-Aps(c-2355) and mouse anti-rabbit IgG-AP (sc-2358) were purchased from Santa Cruz Biotechnology, Inc. The color development Alkaline Phosphatase Conjugate Substrate Kit and all the electrophoresis reagents were from Bio-Rad Laboratories (Hercules, CA, USA). All other reagents (chemicals and solvents) utilized were of analytical grade quality and purchased from standard commercial suppliers.

2.3. Sample preparation

Leaves of laurel and salvilla, aerial parts of arnica, chamomile, marjoram, mint, and yerbaniz, cumin seeds and the DRP without root were milled into powder (40-mesh particle size) and freeze dried. One percent (w/v) infusions were prepared with distilled water maintained at 80°C for 10 min in a hot water bath (Felisa, Fabricantes Feligneo, Jalisco, México). When cooled at room temperature, infusions were centrifuged at 4,500 rpm and the supernatant was filtered through Whatman filter paper No. 1. Aliquots were taken for TPC determinations. The filtrates were frozen and lyophilized in a

freeze dryer (Edward Modulyo) affording the LACE. The LACE were assayed in cell culture, to do so; they were dissolved in DMSO by sonication to a concentration of 300 mg/mL and maintained at −20°C until the experimentation no longer than three months. Just before treatments, extracts were dissolved in the culture medium to the required final concentrations ensuring that the maximum DMSO concentration in medium was less than 0.04%. DMSO was used as control.

2.4. Total phenolic content

The TPC of the nine infusions was determined using the Folin–Ciocalteu method as described by (Ainsworth & Gillespie, 2007). Briefly, the aqueous extracts were oxidized with the Folin–Ciocalteu reagent and then, the reaction was neutralized with 10% sodium carbonate. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 750 nm using a microplate reader (ELISA Daigger). Quantification was performed based on a standard curve with gallic acid monohydrate. Results were expressed as milligrams of gallic acid equivalent per gram of dry weight of extract (µg GAE/mg).

2.5. Deoxy-D-ribose assay (hydroxyl radical scavenging activity)

The antioxidant activity was estimated by the deoxy-D-ribose assay according to the method of Dorman, Bachmayer, Kosar, and Hiltunen (2004) in which the non-site-specific hydroxyl radical-scavenging activity is evaluated. The mixture containing $FeCl_3$ (1.0 mM), ascorbic acid (1.0 mM), EDTA 104 mM, H_2O_2 (1.0 mM), deoxy-D-ribose (2.8 mM) and the tested sample in 500 mL phosphate-buffered saline (PBS 50 mM, pH 7.4) was incubated at 37°C for 1 h. After adding 1 mL of trichloroacetic acid (TCA, 2.8%, w/v) and 1 mL of thiobarbituric acid (TBA, 1.0%, w/v), the reaction mixture was kept for 20 min in a water bath at 100°C. The color development was measured at 532 nm and the scavenging activity of tested samples expressed as the inhibition percentage of the deoxy-D-ribose degradation to malondialdehyde. Results were presented as their half-inhibitory concentrations (IC_{50}).

2.6. High-performance liquid chromatography analysis

Individual phenolic acids and other simple phenolic compounds were identified using an Agilent 1100 model high-performance liquid chromatography (HPLC) with automatic injection that was equipped with an 1100 quaternary gradient pump, in-line degasser, autosampler, dual wavelength UV/vis detector, and acquisition system (Agilent Software 1100, Agilent Technologies Inc., Santa Clara, CA, USA). A 15 × 4.6 mm ID reversed-phase Zorbax octadecylsilane (ODS-C18; Agilent) column was used and operated at room temperature. Phenolic acids were eluted at 1 mL/min using a gradient of two solvent systems: (A) acetic acid–water (2:98 v/v) and (B) acetic acid–acetonitrile–water (2:30:68 v/v). The mobile phase ratio was 90% A and 10% B at time 0; 0% A and 100% B at 30 min. The resolved compounds were detected at 280 nm, identified on the basis of chromatographic retention times of co-eluted pure standards. Six pure phenolic acids (gallic, vanillic, chlorogenic, caffeic, benzoic, and salicylic acids), one flavanone glycoside (hesperidin), as well as four simple polyphenols (catechin, epicatechin, epigallocatechin gallate, and vanillin) were used for calibration and identification.

2.7. Gas Chromatography–mass spectrometry analysis

Some phenolic compounds were identified and quantified by gas chromatography–mass spectrometry (GC–MS). One milli liter of HPLC grade methanol was added to 10 mg of the dry aqueous extracts and sonicated during 1 h, 100 µL of this solution was evaporated with nitrogen gas. 50 µL of BSTFA (*N*,*O*-bis[trimethylsilyl]trifluoroacetamide) derivatizing agent was added prior to stirring at room temperature. Analysis was carried out using an Agilent Series 7890A gas chromatograph (Agilent Technologies, Wilmington, DE, USA), equipped with a HP-5MS capillary column (30 m × 0.5 mm i.d. and coating thickness 0.25 µm) and a linear inert injector Split/splitless (2 mm i.d.). Detection was done with a single quadrupole MS detector 5975C (Agilent technologies) with electron energy set at 70 eV and the mass range at 50–700 *m/z*. Analytical conditions were as follows: injector and transfer line temperature 250°C with a carrier gas helium flow of 1 mL/min; oven temperature, 100°C during 1-min, 220°C at a heating rate of 6°C/min, 290°C per 1.23 min at a heating rate of 10°C/min, and finally to 310°C for 7.5 min at a heating rate of 40°C/min. The flow rate of the carrier gas (helium) was

maintained at 1 mL/min. The CG-MS control and data processing was performed using the software Chem-Station (Agilent technologies). Twelve calibration standards were used: glycerol, vanillin, vanillic acid, cumaric acid, gallic acid, cafeic acid, sinapic acid, resveratrol, epicatechin, catechin, chlorogenic acid, and ellagic acid. Identification of compounds was based on a comparison of the retention times with those of authentic samples with a confidence level higher than 90%. MS spectra of separated compounds were compared with the NIST 08.1 mass spectral database.

2.8. Cell culture

Human colon cancer cell line HT-29 (HTB-38) was purchased from the American Type Culture Collection, grown in RPMI-1640 medium with 10% FSB and 1% penicillin–streptomycin in 100 mm Petri plates, and incubated at 37°C in a humidified atmosphere with 5% CO_2. Cells at 70% of confluence were used for all essays, which were carried out between passages No. 7–14 to ensure uniformity of cell population and reproducibility. The cells were grown in cell culture plates, maintained in RPMI-1640 supplemented with 10% FBS, penicillin (100 units/mL) and streptomycin (0.1 mg/mL). The medium was changed every other day; the cells were subcultured every 4–5 days until they reached 70–80% confluent monolayer. Cells were placed in an appropriate density; exponential growing cells were used in all the experiments. After treatments, cells were harvested by trypsinization and centrifuged (4,500 rpm); cell pellets were then put in phosphate buffer (pH 7.8) supplemented with protease inhibitor (1%), a cell lysis was done with an Ultra-Turrax (IKA T10 basic) and the supernatants were stored immediately at −80°C after centrifuged at 8,500 rpm and 4°C for 15 min. The cell lysate total protein concentration was determined using the NanoDrop 2000 UV/vis spectrophotometer (Thermo Scientific).

2.9. MTT assay (cytotoxicity assay)

Cell mitochondrial enzymatic activity (MEA) was assayed by the 3-(4,5-dimethhylthiazol-2-yl)-2,5-diphenylterazolium bromide (MTT) assay as an indirect measurement of cell viability because the reduction of tetrazolium salts is widely accepted as a reliable method to examine cell viability/proliferation. Cells were seeded in 96-well plates at an initial density of 9.6×10^3 cells/well in RPMI-1640. Briefly, after 72 h extracts were tested at eight final concentrations (0.125, 0.25, 0.75, 1.25, 2.5, 5.0, 7.5, and 10 mg/mL) dissolved in SFB-free medium. All controls received appropriated carriers; DMSO was kept to a maximum concentration of 0.04%. Assay was carried out in six fold to analyze the cytotoxicity kinetics for each extract and determine the concentration to be used in later assays. Cells were treated with extracts for 24 h after each well was washed twice with PBS (1X) to remove extract residues. MTT solution was added and plates were incubated for 4 h. The formazan crystals formed were dissolved in 200µL of DMSO. The resulting absorbance was read at 570 nm (measurement) and 690 nm (reference) using a 96-well plate reader (ELISA, Daiger).

2.10. Western blotting

Expression of COX-1, COX-2, TNFα, total NF-κB, and IL-8 was measured by western blot technique using specific monoclonal antibodies. Cells treated with the extracts were used. Proteins (70 µg) were separated by SDS-PAGE electrophoresis gels and transferred to nitrocellulose membranes blocked during 12 h with Svelty® milk 5%w/v in TBS 1X. Membranes were incubated at 4°C at low agitation during 3 h with the antibodies under the specific concentrations: COX-1 (1:1,000), COX-2 (1:1,000), TNFα (1:200), NF-κB (1:200), IL-8 (1:200), and GAPDH (1:800) followed by incubation with alkaline phosphatase-conjugated secondary antibodies in a dilution range 1:4,000–1:5,000 (anti-mouse for COX-1, COX-2, and TNFα; anti-rabbit for NF-κB, IL-8, and GAPDH). Proteins were visualized by using the color development kit specifications.

2.11. Statistical analysis

Data were analyzed by a one-way analysis of variance (ANOVA). Significant differences among treatment groups were evaluated by Fisher test ($p < 0.05$) and expressed as mean ± SD ($\alpha = 0.05$, Tukey). All statistical analyses were made using STATISTICA 7 software.

3. Results

3.1. TPC of infusions

Plants produce an extraordinary diversity of phenolic metabolites. An important source of these phytochemicals in the human diet could come from herbal infusions (Atoui et al., 2005). The TPC measured by the Folin–Ciocalteu method on the herb infusions prepared to a concentration of 1% (w/v) is shown in Table 1 that was calculated in dry basis. The lowest TPC values were found in DRP (8 µg GAE/mg) and cumin (15.3 µg GAE/mg) that are also around the ones reported by Sánchez-Burgos et al. (2013) for *Quercus* species, which have demonstrated gastroprotective effect; they found 6.98 µg GAE/mg (*Q. obtusata*) and 17.26 µg GAE/mg (*Q. resinosa*). The highest TPC was found in laurel (70.7 µg GAE/mg), but still is being significantly low compared with the values reported for Chinese green tea (*C. sinensis*) by Atoui et al. (2005) of 405.33 µg GAE/mg. Atoui et al. (2005) also studied chamomile and mint, reporting 35.33 µg GAE/mg for both, while we found 27.3 and 19.27 g GAE/mg respectively. Polyphenols are produced in all plants as part of their secondary metabolism; formation of bioactive constituents of plants depends on the actual environmental and growing factors. Application of specific stress, such as drought stress can optimize the production of secondary plant metabolites by inducing natural biochemical changes in plants (Manukyan, 2011), reason why the TPC will vary depending on where and when the plants were grown and harvested. The age and the storage conditions of the plant material are also of great significance for maintaining high quality standards. Additionally, the studied plants are local ones and it is not possible to compare them with data from a specific reference or database.

3.2. Antioxidant activity of the LACE

Considerable attention has been focused on exploring the potential antioxidant properties of plant extracts or isolated products of plant origin. The hydroxyl radical is an extremely reactive oxygen species capable of modifying several biologically important molecules in the living cells. *In vitro* hydroxyl radicals are generated by a mixture of ascorbic acid, H_2O_2, and Fe^{III}-EDTA. The hydroxyl radicals that are not scavenged by other components of the reaction mixture react with the deoxy-D-ribose sugar and cause its degradation into a series of products, some of which react on heating with TBA at low pH to give a chromogenic compound. The results of scavenging capacity of hydroxyl radicals are presented in Table 1. We determined the IC_{50} of catechin (1,033.05 µg/mL) and gallic acid (1,478.12 µg/mL), which were used as positive controls. The relative order of efficiency was: catechin > gallic acid > DRP > arnica > yerbaniz > salvilla > marjoram > cumin > mint > chamomile > laurel. There is no correlation between TPC and the IC_{50} of extracts. This could be because the TPC assay detects a variety of phenolic compounds that go from simple phenolic acids, tannins, and flavonoids *inter alia* and the deoxy-D-ribose assay has more affinity for flavonoids, which are a different proportion of the TPC in each sample.

Table 1. Yield, TPC, and IC_{50} of lyophilized aqueous crude herbal extracts

Sample	Yield (%)	TPC (µg GAE/mg)	IC_{50} (µg/mL)
Arnica	21.03 ± 3.05^{abd}	26.2 ± 5.32^{ab}	$2{,}559.81 \pm 11.77^{ab}$
Chamomile	34.21 ± 4.27^{f}	27.3 ± 6.85^{b}	$4{,}713.27 \pm 8.90^{bc}$
Cumin	25.84 ± 1.50^{be}	15.3 ± 4.24^{ac}	$4{,}200.31 \pm 25.91^{abc}$
DRP	13.59 ± 1.20^{c}	8.0 ± 4.10^{c}	$2{,}452.53 \pm 0.09^{a}$
Laurel	21.82 ± 0.78^{ab}	70.7 ± 2.73^{d}	$5{,}097.11 \pm 5.11^{c}$
Marjoram	13.92 ± 1.25^{cd}	66.8 ± 1.90^{d}	$4{,}097.80 \pm 6.93^{abc}$
Mint	17.75 ± 1.29^{acd}	19.2 ± 0.95^{abc}	$4{,}507.57 \pm 11.24^{abc}$
Salvilla	20.31 ± 0.61^{abcd}	23.3 ± 1.19^{ab}	$2{,}947.43 \pm 31.88^{abc}$
Yerbaniz	22.25 ± 2.36^{ab}	44.3 ± 2.50^{e}	$2{,}918.56 \pm 5.05^{abc}$

Significant statistical differences were determined by Fisher ($p < 0.05$) after the ANOVA test.

3.3. Phenolic compounds detected by HPLC and GC–MS

Due to the diversity and complexity of the mixtures of antioxidant compounds in the different plant extracts, it is quite difficult to identify every compound. Phenolics are compounds possessing one or more aromatic rings with one or more hydroxyl groups and generally categorized as phenolic acids, flavonoids, stilbenes, coumarins, and tannins (Liu, 2004). The phenolic composition performed by HPLC is presented in Table 2 and the one carried out by GC–MS is shown in Table 3. Few phenolic acids, hesperidin, vanillin, and EGCG were detected by HPLC. Salvilla and DRP are the scarcer regarding compounds detected by GC–MS, while cumin, chamomile, and mint are the richest regarding these types of compounds. However, global antioxidant activity of extracts may be attributed to synergic and additive interactions. Thousands of phytochemicals are present in whole food. These compounds differ in molecular size, polarity, and solubility, which may affect the bioavailability and distribution of each phytochemical in different macromolecules, subcellular organelles, cells, organs, and tissues. This balanced natural combination present in fruits and vegetables cannot simply be mimicked by pills or tablets (Liu, 2004).

3.4. Effect of aqueous extracts on cell cytotoxicity

The MTT assay was performed to make a preliminary assessment of the growth inhibitory potential of extracts on HT-29 cells at 24 h of treatment. The modification of medium pH at different concentrations of the baseline was measured, not finding any modification regarding the controls. After 24 h, the stability (precipitation) of extracts was documented by pictures finding a direct correlation with the MTT results. Treatment with extracts exhibited a decrease in the MEA in a dose-dependent manner as shown in Figure 1. The results for most of the LACEs showed a similar behavior at doses lower than 0.75 mg/mL for chamomile, salvilla, and yerbaniz. At doses lower than 2.5 mg/mL, cumin, DRP, laurel, marjoram, and mint showed a slight increase in the MEA, this increment may be due to the nature of extracts, which might provide some vitamins and nutrients to the cells. The LACE that had not a major effect on this parameter (MEA 80–100%) were laurel and yerbaniz from 0.75 to 1.25 mg/mL, chamomile at 1.25 mg/mL, cumin, DRP, and marjoram from 2.5 to 5.0 mg/mL and mint at 5.0 mg/mL. The extracts that caused a decrease in the MEA over 80% were salvilla at concentrations of 1.25 mg/mL and up; laurel and yerbaniz from 2.5 mg/mL and up; chamomile from 5.0 mg/mL and up; and cumin, DRP, marjoram, and mint from 7.5 mg/mL and up. As it is noticeable, arnica

Table 2. Phenolic compounds detected by HPLC in lyophilized aqueous herbal extracts

Phenolics (retention time*)	Arnica	Cumin	DRP	Mint	Laurel	Chamomile	Marjoram	Salvilla	Yerbaniz
Hesperidin (6.56)	−−−	+++	+++	+++	−−−	−−−	−−−	+++	−−−
Cumaric acid (7.52)	+++	−−−	+++	+++	+++	+++	−−−	−−−	+++
Vanillic acid (19.87)	+++	+++	+++	+++	+++	+++	−−−	+++	−−−
Caffeic acid (20.52)	+++	−−−	+++	−−−	−−−	+++	+++	+++	−−−
EGCG (21.93)	+++	+++	+++	+++	+++	+++	−−−	+++	−−−
Vanillin (26.42)	+++	+++	+++	+++	+++	+++	+++	+++	+++
Rosmarinic acid (30.78)	−−−	−−−	−−−	−−−	−−−	−−−	+++	−−−	−−−
Chlorogenic acid (2.82)	+++	−−−	−−−	−−−	−−−	−−−	−−−	−−−	−−−

*Retention time is given in minutes; −−− absence, +++ presence.

Table 3. Phenolic compounds detected by GC–MS in lyophilized aqueous herbal extracts

Phenolic (retention time*)	Arnica	Cumin	DRP	Mint	Laurel	Chamomile	Marjoram	Salvilla	Yerbaniz
Benzoic acid (6.390)	---	---	---	0.279	---	0.393	0.282	---	---
Salicylic acid (11.368)	---	---	---	0.274	---	---	0.203	---	---
4-Hydroxybenzoic acid (13.486)	0.569	0.281	---	0.187	---	0.191	0.131	---	0.436
Gentisic acid (16.322)	---	---	---	---	---	0.227	---	---	---
Protocatechuic acid (17.039)	1.636	0.103	---	0.993	0.115	0.330	0.244	---	2.127
Vanillin (11.824)	0.225	1.156	---	---	0.488	---	0.123	---	0.397
Vanillic acid (16.048)	1.957	0.267	0.436	0.541	0.231	0.984	0.119	0.626	0.196
Gallic acid (19.431)	0.105	0.145	---	---	---	---	---	---	0.059
Tyrosol (12.465)	---	---	---	---	---	---	0.085	---	---
Phenylacetic acid (7.261)	0.181	---	---	---	---	---	---	---	---
4-Hydroxyphenilacetic acid (13.725)	---	0.071	---	0.055	---	0.032	---	---	---
α-Hydroxycinnamic acid (12.797)	---	0.093	---	---	---	---	---	---	0.631
Cumaric acid (18.918)	0.113	0.104	---	0.106	0.143	0.276	---	---	---
o-Cumaric acid (16.415)	---	---	---	0.417	---	---	---	---	---
Caffeic acid (22.147)	0.164	0.097	---	0.866	---	0.358	0.188	0.480	0.226
Ferulic acid (21.344)	0.056	0.114	---	0.063	0.126	0.130	---	---	---
Umbelliferone (17.159)	---	---	---	---	---	0.695	---	---	1.208
Chlorogenic acid (35.988)	0.069	---	---	---	---	---	---	---	---
Rosmarinic acid (38.565)	---	---	---	0.568	---	---	1.192	---	---
Ellagic acid (37.368)	---	0.020	---	---	---	---	---	---	---
Catechin (33.934)	---	0.017	---	---	---	---	---	---	---
Epicatechin (33.678)	0.036	0.106	---	0.377	0.500	---	0.025	---	---

*Retention time is given in minutes; Concentration is expressed as relative area (%).

showed a remarkable cytotoxicity decreasing the MEA 40% even at the lowest concentration of 0.125 mg/mL. In order to use the same concentration for all extracts, the lowest dose (0.125 mg/mL) was chosen to continue the experimentation.

3.5. LACE effect on inflammation markers by western blot

Western blot analyses were carried out both in the LACE (at 0.125 mg/mL, 3 h) and with indomethacin (250 μM, 6 h) followed by LACE under the aforementioned conditions (Figure 2(A–L)). GAPDH was used as an internal control for equal protein loading (Figure 2 (E and F)).

3.6. LACE effect on COX expression

COX-1 is constitutively expressed in many tissues, but the expression of COX-2 is regulated by mitogens, tumor promoters, cytokines, and growth factors. Several researchers have reported that many dietary polyphenols inhibit COX activity at the transcriptional level as well the enzymic level (Aggarwal & Shishodia, 2006).

The adverse effects attributable to NSAIDs are thought to arise from the inhibition of the constitutive isoform of the target enzyme COX-1. Under the conditions mentioned above in 2.5 we observed (Figure 2(A)) that COX-1 expression was not inhibited by any of the LACE, it is also expressed in the negative control with DMSO as a constitutive enzyme in HT-29 cell line, but it was inhibited by indomethacin[Q Edits]. In Figure 2(B) corresponding to the curative treatment we can see that all

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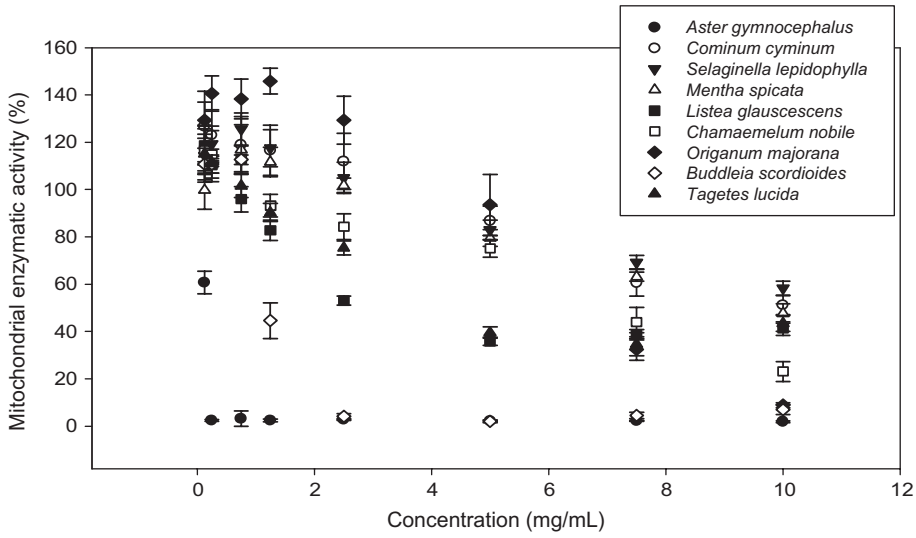

Figure 1. Effect of infusions on HT-29 cells determined by the MTT assay.

Notes: Cells were treated with the LACE at different concentrations (0.125, 0.25, 0.75, 1.25, 2.5, 5.0, 7.5, and 10 mg/mL) during 24 h. The mitochondrial enzymatic activity (MEA %) was determined comparing with the control (DMSO 0.04%).

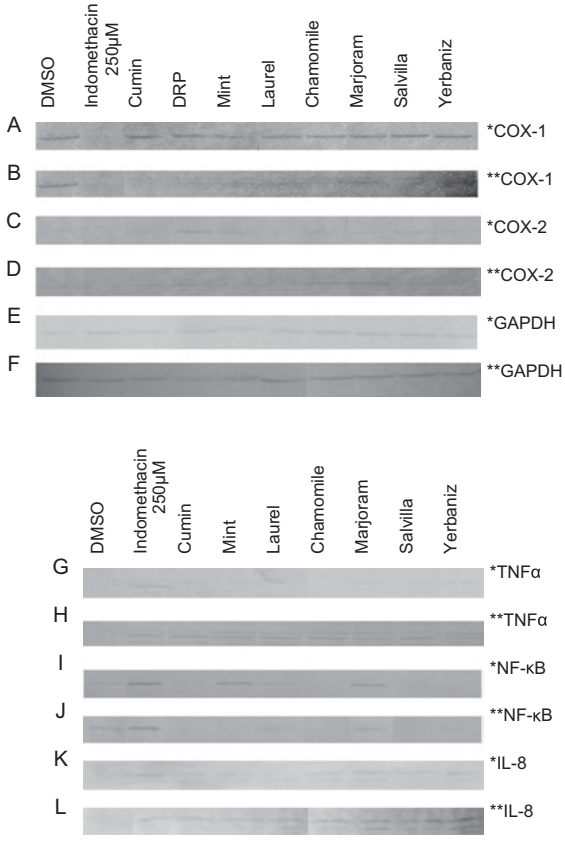

Figure 2. Expression of COX-1, COX-2, TNFα, NF-κB, and IL-8 by western blotting using GAPDH as a protein loading control.

*Treatment with LACE at 0.125 mg/mL; ** Treatment with indomethacin 250 μM followed by the LACE at 0.125 mg/mL.

the LACE, except the cumin extract, have the capacity to regenerate the damage caused by indomethacin achieving a detectable expression of COX-1 after the enzyme was suppressed by indomethacin.

COX-2 is an immediate-early gene thought to be involved in inflammation. In Figure 2(C) we observe that COX-2 is expressed in the DMSO control, this is due to the own nature of the cell line that we are working with, the light expression corresponds to the basal levels in an adenocarcinoma cell. Pro-inflammatory cytokines contribute to increased COX-2 protein levels during the inflammation response. Oncogenes, growth factors, cytokines, chemotherapeutics, and tumor promoters are among some of the stimuli that induce COX-2 expression. The promoter region of COX-2 consists of many transcription factor binding sites, such as NF-κB (Brown & DuBois, 2005). It is remarkable that the DRP samples over express COX-2 compared to the control (Figure 2(C)), which could be a possible inflammatory response in our system. The rest of the LACE just expresses COX-2 as the control does. The expression of COX-2 on the curative treatment corresponds to the baseline on the rest of the extracts regarding the DMSO control (Figure 2(D)).

3.7. LACE effect on TNFα expression

Western blot analysis of TNFα expression with the LACE shown in Figure 2(G) expose light expression of the cytokine in all cases including DMSO control but shows a clear stronger expression in the indomethacin lane, which corresponds to literature reports on the indomethacin activating TNFα signaling pathway by increasing ROS (Giardina et al., 1999). In our study we found chamomile to be the extract that best modulate TNFα despite the effect of salvilla after activating TNFα with indomethacin. It is important to stand out herein that Chamomile had the capacity to inhibit TNFα expression to non-detectable levels by this technique. After indomethacin has induced oxidative stress TNFα is expressed in all cases (Figure 2(H)), which might be due to the short exposure time of the treatments not allowing the cells to repair the effect caused by indomethacin over TNFα expression.

3.8. LACE effect on NF-κB expression

NF-κB is activated by free radicals, inflammatory stimuli, cytokines, carcinogenesis, tumors promoters, endotoxins, γ-radiation, ultraviolet light, and X-rays (Krishnaswamy, Devaraj, & Padama, 2010). Several dietary agents have been found to be potent inhibitors of NF-κB which may block any one or more steps in the NF-κB signaling cascade, translocation of NF-κB into the nucleus, DNA binding of the dimers, or interactions with the basal transcription machinery (Aggarwal & Shishodia, 2006). NF-κB plays a pivotal role in chronic and acute inflammatory diseases (Karin, Yamamoto, & Wang, 2004). In our study we found an expected similar behavior of NF-κB with TNFα in most of the cases, which is due to the fact that the first one can be activated by the second one (Figure 2(I)). Regarding this nuclear factor, the best modulation was presented by cumin, chamomile, salvilla, and yerbaniz while marjoram and mint did not give the best modulation effect. In the case of marjoram and mint, NF-κB is more expressed than in the controls and the rest of herbs, which suggests different activation mechanisms of the nuclear factor that is not by TNFα. In Figure 2(J) we observed that despite that mint overexpressed NF-κB when was applied alone, when it was applied after indomethacin, the extract decreased the expression of this inflammation marker, which probably indicates that mint extract may be acting directly over the ROS produced by indomethacin rather than over NF-κB. Nevertheless, this was not the case of marjoram that showed no decrease in the expression of NF-κB even after applying indomethacin[Q edits]. Besides mint and marjoram, all the other LACE showed a modulation effect by decreasing NF-κB expression with respect to the indomethacin control and even with respect to the baseline of the DMSO control.

3.9. LACE effect on IL-8 expression

IL-8 is an important mediator of inflammation that can be induced by reactive oxygen species and suppressed by antioxidants in cell-type specific fashion. Expression of IL-8, the major human neutrophil chemoattractant and inflammatory mediator, is dependent on IL-1β activation of NF-κB.

The best modulation of IL-8 was observed by laurel (Figure 2(K)) in which this extract decreased the expression of IL-8 to a not detectable level. The extracts did not have a noticeable curative effect after applying the indomethacin (Figure 2(L)). Kogiannou, Kalogeropoulos, Kefalas, Polissiou, and Kaliora (2013) demonstrated the modulation of IL-8 by some herbal infusions in HT-29 cells stimulated with TNFα. In their survey they try the extracts for 24 h and found a reduction in the expression

of IL-8 in the case of chamomile and marjoram; in our study the stimulation was a step behind with indomethacin trying to simulate what population does by consuming NSAIDs and the treatments where for only 3 h, reason why we could not see a noticeable effect over this cytokine.

4. Discussion

The term oxidative stress refers to the situation of serious imbalance between production of reactive species and antioxidant defense. The resulting increase in the oxidative damage of biomolecules may play an important role in the pathology of several human diseases, therefore it would be amenable to the therapeutic intervention with appropriated antioxidants (Halliwell, 2001). Trying to find these appropriated compounds from natural sources and using the empirical knowledge, we have evaluated the infusions of some herbs traditionally used for gastrointestinal disorders, which most of the times involve oxidative stress.

Cellular response to oxidative stress is dependent upon the enzymatic and non-enzymatic antioxidant defenses in the cells, the type of oxidant and the nature of oxidative stress induced, as well as dose and exposure times. Cells effectively combat oxidative stress when there is a balance between these factors (Kalaiselvi, Rajashree, Bharathi Priya, & Padma, 2013). A problem comes out when this balance yields in favor of the oxidants. The consumption of NSAIDs is one reason why an unbalance happens. It stimulates ROS generation in the gastrointestinal tract. Same thing happens with the human colon cancer HT-29 cell line, since indomethacin is an NSAID. This drug exhibits its secondary effects on the cell by inducing oxidative stress and up regulating inflammatory genes. That is why it was used in our model, where it effectively showed an increasing expression of pro-inflammatory markers. Components with anti-inflammatory effects that are able to selectively inhibit COX-2 without inhibiting COX-1 are desirable as we found in eight of the nine LACEs, because only DRP could not reduce the expression of COX-2, although it did not modulate COX-1 either. COX-2 is a well-known NF-κB target involved in inflammation. In colon epithelial cells, COX-2 can be induced by TNFα in a time-and concentration-dependent manner. Wright et al. (2004) demonstrated that TNFα induces COX-2 within 3 h. Acute production of COX-2 is likely to be pro-inflammatory, so the partial inhibition of COX-2 could be regarded as a beneficial outcome in terms of inflammation. It has been demonstrated using HT-29 intestinal epithelial cells that TNFα induction of COX-2 is completely dependent on the transcription factor NF-κB (Wright et al., 2004).

The induction of pro-inflammatory genes by TNFα has been linked to most diseases. The pro-inflammatory effects of TNFα are primarily due to its ability to activate NF-κB. Almost all cell types, when exposed to TNFα, activate NF-κB, leading to the expression of inflammatory genes, which include COX-2, lipoxygenase-2 (LOX-2), cell-adhesion molecules, inflammatory cytokines, chemokines, and inducible nitric oxide synthase (iNOS) (Aggarwal & Shishodia, 2006). In our experimentation we went back to the activation of TNFα by indomethacin. Once TNFα is stimulated, it will activate NF-κB, which is clearly one of the most important regulators of pro-inflammatory gene expressions. Following this signaling pathway, we can explain why a remarkable modulation of COX-2, TNFα, and IL-8 was not observed in our study. By trying the activation of NF-κB directly by TNFα probably would expose a positive modulation, but going back to the activation of TNFα by indomethacin and having 3-h exposure time of cell to LACE we could not see the inhibitory effect over COX-2 as Wright et al. (2004) reported previously.

De novo polyphenols seem to be important metabolic modulators by virtue of their ability to moderate and influence several cellular processes such as signaling, proliferation, apoptosis, redox balance, differentiation, etc. In fact, polyphenols may be perceived as future pharmacological agents and may be used as antioxidant and anti-inflammatory enforcements to combat oxidative challenges (Rahman et al., 2006). The phenolic composition from our plant infusions, according to the HPLC analysis, presented considerable variations on their phenolic profiles due to the nature of the plant materials used. Even there is little information about the chemical composition of most plants studied in this work; some of the phenolic compounds identified here have been previously reported

in organic extracts or aqueous extracts for these plants. For example, Carnat, Carnat, Fraisse, Ricoux, and Lamaison (2004) also identify caffeic acid in *C. nobile* flowers infusion.

In order to extend the profile of bioactive compounds a GC–MS analysis was performed. Low molecular weight compounds were identified, which could be related to the method used. Some of the compounds identified in several herbal infusions were also identified by HPLC analysis. For example, vanillic acid was identified in arnica (*A. gymnocephalus*) by HPLC and also as the major compound quantified by GC–MS. This was also the case of rosmarinic acid, which was observed in marjoram infusion (*O. majorana*). On the other hand, authors such as Ani, Varadaraj, and Akhilender Naidu (2006) have reported a similar phenolic compound profile as we have for cumin (*C. cyminum*), whereas in plants such as desert resurrection plant (*S. lepidophylla*), laurel (*L. glaucescens*, and salvilla (*B. scordioides*) we believe this may be the first report on such plant chemical composition.

Even though we have not observed a great relation between TPC and antioxidant capacity, the affluence of phenolic compounds detected by HPLC and GS–MS and the modulation of pro-inflammatory targets may suggest that the phenolic fraction is in part responsible for the biological activity of these herbal infusions. Several studies have reported potential effects of some phenolic compounds like the ones identified in this work. For example, Tai, Sawano, Yazama, and Ito (2011) reported antioxidant capacity for vanillic acid using multiple antioxidant assays. Though, vanillic acid was the main compound identified in arnica, cumin, DPR, chamomile, and salvilla infusions, they have shown variable results in antioxidant values. Therefore, we may assume that the antioxidant capacity of these samples is not only dependent on the polyphenol content, but also in the synergy with other phytochemicals present. Certainly more studies in this direction are needed.

A proper understanding of how polyphenols are absorbed and transformed pre- and post-absorption is helpful in understanding how *in vitro* observation can be translated into the *in vivo* context. In recent years there has been a remarkable increment in scientific knowledge dealing with the beneficial role of polyphenols during oxidative stress. Nonetheless, the additive and synergistic effects of phytochemicals in fruits and vegetables have been proposed to be responsible for their antioxidant and anticancer activities (Liu, 2004). This may partially explain why no single or universal antioxidant could replace the combination of natural phytochemicals in achieving the observed health benefits (Vinothkumar & Nalini, 2013).

The IC_{50} for DRP was the lowest one, i.e. the best antioxidant response. We could observe that this extract is the only one that over expresses COX-2 comparing with the control. This indicates that the main compounds present in DRP, which have a high capacity of scavenging hydroxyl radicals, might cause an inflammatory response activated by COX-2. In the case of arnica, the second top IC_{50} in the deoxy-ribose test, showed a high cytotoxicity, while the infusions with higher IC_{50} showed better results in the inflammatory markers as well as less cytotoxicity in the MTT assays.

The use of cell cultures as alternatives to predict antioxidant effects of certain samples is the background of *in vivo* studies. Several researches have demonstrated the correlation between *in vitro* and *in vivo* results (Lee et al., 2005; Sato et al., 2011). However, many of those studies did not take bioavailability and metabolism factors into consideration, and the effects reported in those studies do not necessarily occur *in vivo*. Although most phenolics are absorbed to some extent, this is very dependent on the type of polyphenol. The range of concentrations required for an effect *in vitro* varies from <0.1 μmol/L to >100 μmol/L. Because physiologic concentrations do not exceed 10 μmol/L, the effects of polyphenols *in vitro* at concentrations of >10 μmol/L are generally not valid, with the possible (but unproven) exception of the intestinal lumen (Williamson & Manach, 2005). According to Figure 1, it would not be recommendable to intake large amounts of herbal infusions since they might be cytotoxic. Therefore, more studies for herbal infusions are needed in order to determine their eventual effect in humans.

5. Conclusions

The LACE of salvilla, chamomile, and laurel had a positive effect over pro-inflammatory markers, relieving oxidative stress and down regulating COX-2, TNFα, NF-κB, and IL-8. The present study demonstrates that some of the phenolic extracts do have an effect on the inflammatory markers by themselves and even after the cytotoxic effects of indomethacin in HT-29 cells. These results suggest that analyzed extracts act as anti-inflammatory agents, which throws light on the traditional intake of these herbal infusions as an alternative treatment for intestinal inflammation.

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