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Gastroprotection as an example: Antiadhesion against *Helicobacter pylori*, anti-inflammatory and antioxidant activities of aqueous extracts from the aerial parts of *Lippia integrifolia* Hieron

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ABSTRACT

Ethnopharmacological relevance: The aerial parts of *Lippia integrifolia* (Gris.) Hieronymus (Verbenaceae), known as *incayuyo*, are used by the peasant population of Argentina for treatment of diseases related to a gastrointestinal system, mainly for inflammation of the stomach and have also been included into the Argentina Food Code. This study aimed to investigate the phytochemical profile of hydrophilic extracts from the herbal material by LC–MS and to evaluate potential pharmacological mechanisms rationalizing the traditional use of *incayuyo* aqueous extracts potential anti-inflammatory treatment of gastrointestinal disorders.

Materials and methods: Phytochemical profiling: LC–MS of an aqueous decoction. Antiadhesive effects against *Helicobacter pylori*: *in vitro* FACS assay using FITC-labeled bacteria and AGS human stomach cells. Influence of extracts on stomach cells and RAW 264.7 macrophages: MTT viability assay and BrdU proliferation ELISA. Influence of extracts on IL-6 and IL-8 secretion from stomach cells was quantitated by ELISA after infection of the cells with *Helicobacter pylori*. Influence of test extracts on macrophages: phagocytosis of FITC-labeled Zymosan particles and NO production. Antioxidative capacity: DPPH assay and O_2-induced caroten oxidation.

Results: LC–MS profiling indicated the presence of compounds **1**–**20** with flavonoid hexosides, phenylethanoides (acteoside, isoacteoside) and sesquiterpenes [(epi)lippidulcine, peroxylippidulcine] in the decoction extract and subfraction PhF. The extract exhibits strong *in vitro* antioxidative capacity and inhibited adhesion of *Helicobacter pylori* to stomach cells up to 40%, while an EtOH-soluble fraction showed inhibition rates of up to 60%. Decoction increased the cellular viability of AGS cells significantly at >10 µg/mL, while proliferation rate was not influenced. *Helicobacter pylori* induced IL-8 secretion was significantly reduced by coincubation of AGS cells with the extracts. Aqueous extracts stimulated phagocytosis rate of macrophages and inhibited the LPS-induced NO-secretion.

Conclusions: The traditional use of aqueous extracts from *Lippia integrifolia* for gastric inflammation seems to be rationalized: besides anti-inflammatory effects on stomach cells antiadhesive properties of the extracts against the main bacterial inductor of gastritis *Helicobacter pylori* were identified. Additional effects for stimulation of innate immunity and potential radical scavenging effects may additionally contribute to the activity of the extracts.

1. Introduction

Lippia integrifolia (Gris.) Hieronymus (Verbenaceae) is a woody aromatic shrub, reaching 1–1.5 m high and growing in rural areas of Bolivia and in northwestern and center of Argentina. The species is well known in La Rioja, San Juan, Catamarca, Salta, Tucumán and Córdoba provinces as *pulco, inca yerba, poleo, te del inca, manzanillo* and more commonly as *incayuyo*. Traditionally

Abbreviations: BHT, Butylhydroxytoluol; FCS, fetal calf serum; PhF, phenolic fraction, polysaccharide-depleted low molecular weight material from aqueous extract; RPS, raw polysaccharides

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incayuyo, prepared from the aerial parts (leaves and flowers), was used by the peasant population for treatment of diseases related to a gastrointestinal system, mainly for inflammation of the stomach and dyspepsia (Alonso and Desmarchelier, 2006). Additionally *incayuyo* has also been documented as diuretic, emmenagogue and antibiotic remedy and for treatment of cough (Ratera and Ratera, 1980; Rondina et al., 2003). Traditionally hot aqueous extracts in form of infusions or decoctions are used. Besides, in Argentina some commercial beverages from *incayuyo* are known as aperitif and tea preparations which are included in the Argentina Food Code (2013).

From the phytochemical point of view different studies have been carried on *incayuyo*, mainly related to the composition of its essential oil, which contains unusual lippiafoliane (tricyclo[5.4.0.02,4]-undecane ring system, Cerda-García-Rojas et al., 2005), africanane sesquiterpenes (Catalán et al., 1991, 1992, 1993, 1994, 1995; Fricke et al., 1999; Cerda-García-Rojas et al., 2005, 2008) and bisabolan-type sesquiterpenes (Ono et al., 2005).

From the functional point of view the essential oil from *Lippia integrifolia* was shown to exert antifungal activity, i.g. against *Aspergillus* spp. (Bluma et al., 2008; Bluma and Etcheverry, 2008), but showed a limited activity against dermatophytes (Muschietti et al., 2005). The use of the volatile oil as moderately active repellent against mosquitoes has been described (Gleiser et al., 2011). However, only few reports on the biological properties concerning the traditionally described gastrointestinal activity have been published. Gorzalczany et al. (2008) evaluated the choleretic and antispasmodic activity of *incayuyo* aqueous extracts in rats and correlated respective the observed effects with mono- and dicaffeoylquinic acids with known choleretic and hepatoprotective activities (Speroni et al., 2003).

The present study aimed to investigate the phytochemical profile of hydrophilic extracts from the herbal material by chromatographic mass spectrometric profiling and to evaluate potential pharmacological mechanisms rationalizing the traditional use of *incayuyo* aqueous extracts potential anti-inflammatory treatment of gastrointestinal disorders.

2. Materials and methods

2.1. General experimentation procedure

If not stated otherwise all chemicals were purchased from VWR (Darmstadt, Germany).

2.2. Plant material and extraction

Aerial parts (leaves, flowers) from *Lippia intergrifolia* were collected in Ampimpa, Tucumán, Northwest Argentina (26°36′10.86″S; 65°50′49.63″O; 2434 m over sea level) during the flowering season from February to March 2009 and identified by Prof. Dr. Catalán C.A.N. (INQUINOA-CONICET, Organic Chemistry Institute, Faculty of Biochemistry, Chemistry and Pharmacy, National University of Tucumán, Argentina). Voucher samples (LIL60687) were deposited in the Herbarium of Miguel Lillo Institute, Tucumán, Argentina.

The samples were dried at room temperature for 7 days under light protection. The leaves and flowers were separated from the stems and pulverized.

For preparation of infusion 15 g of pulverized material were added into 150 mL of boiling distilled water. The mixture was allowed to cool down for 2 h to room temperature, followed by filtration (Whatman® paper, No. 1) and subsequent lyophilization. Yield of the slightly brownish product was 3.6 g (24% w/w, related to the dry weight of starting material).

For preparation of the decoction 15 g of pulverized material were added into 150 mL of distilled water. The suspension was boiled at 100 °C for 2 h under reflux, cooled down to room temperature, filtered and lyophilized. Yield of the slightly brownish product was 3.7 g (25% w/w, related to the dry weight of starting material).

2.3. Isolation of raw polysaccharides (RPS)

RPS were isolated from the decoction extract. 1 g of lyophilized decoction (see Section 2.2) was dissolved in 20 mL of distilled water. To precipitate the polysaccharides, the solution was dropped into 80 mL ethanol 96% at 8 °C over a 16 h time interval, pelleted by centrifugation at 3000*g*, dissolved in water, reprecipitated again in cold ethanol, and dialyzed for 3 days against distilled water (cellulose membranes, MWCO 3.5 kDa, Roth, Karlsruhe, Germany). Yield of RPS obtained after lyophilization was 0.094 g (9.4%, related to lyophilized decoction extract, 2.3%, related to the dried plant material).

The hydroethanolic solution, used for precipitation of RPS was separated from insoluble high molecular weight material, evaporated and lyophilized to yield 0.72 g of a brownish product, named *phenolic fraction* (PhF).

2.4. Analysis of carbohydrate composition of RPS

The analysis was performed according to methods described in detail by Sehlbach et al. (2013) and Hermann et al. (2012).

2.5. Quantification of phenolic compounds and flavonoids

Phenolic compounds were quantified by Folin–Ciocalteu reagent according to Singleton et al. (1998). Extracts were dissolved in water as stock solution (4 mg/mL). Serial dilutions (0.5 mL) were mixed in glass tubes with 2.5 mL Folin–Ciocalteu phenol regent (Merck, Darmstadt, Germany), and 2 mL sodium carbonate (Anedra, Argentina) solution 7.5%. The solution was made up with water to 5.0 mL, mixed, incubated at room temperature for 30 min and absorbance was determined at $\lambda = 725$ nm (UV–visible 160A, Shimadzu, Japan). Phenol content was calculated against gallic acid (> 98%, Sigma, Steinheim, Germany).

Flavonoids interact with aluminum chloride, forming a stable complex which absorbs at $\lambda = 385$–440 nm. According to Arvouet-Grand et al. (1994) 1.5 mL of serial dilutions of the extract stock solution (4 mg/mL) were mixed with 1.5 mL of a solution of aluminum chloride (Anedra, Argentina) in methanol (2% w/v). After 10 min incubation at room temperature the absorbance was determined at $\lambda = 415$ nm. Quantification was made by calibration using quercetine (> 99%, Riedel-de Haen, Seelze, Germany) in the concentration range from 4 to 7 μg/mL.

2.6. Phytochemical profiling of the decoction by UHPLC-DAD-qTOF-MS

Chromatographic separation of the decoction extract was performed on a Dionex Ultimate 3000 RS Liquid Chromatography System on a Dionex Acclaim RSLC 120, C18 column (2.1 × 100 mm^2, 2.2 μm) with a binary gradient (A: water with 0.1% formic acid; B: acetonitrile with 0.1% formic acid) at 0.4 mL/min: 0–5 min: isocratic 5% B; 5–37 min: linear from 5% B to 100% B; 37–47 min: isocratic 100% B; 47–48 min: linear from 100% B to 5% B; 48–55 min: isocratic 5% B. The injection volume was 2 μL. Eluted compounds were detected using a Dionex Ultimate DAD-3000 RS over a wavelength range of 200–400 nm and a Bruker Daltonics micrOTOF-QII time-of-flight mass spectrometer equipped with an Apollo electrospray ionization source (Bruker micrOTOF QII,

Bremen, Germany) in positive mode at 2 Hz over a mass range of m/z 50–1000 using the following instrument settings: nebulizer gas nitrogen, 4 bar; dry gas nitrogen, 9 L/min, 200 °C; capillary voltage 4500 V; end plate offset −500 V; transfer time 70 μs; collision gas nitrogen; collision energy and collision RF settings were combined to each single spectrum of 1000 summations as follows: 250 summations with 20% base collision energy and 130 Vpp+250 summations with 100% base collision energy and 500 Vpp+250 summations with 20% base collision energy and 130 Vpp+250 summations with 100% base collision energy and 500 Vpp. Base collision energy was 50 eV for precursor ions with a m/z less than 500 and then linearly interpolated against m/z up to a maximum of 70 eV for precursor ions with a m/z of up to 1000. Internal dataset calibration (HPC mode) was performed for each analysis using the mass spectrum of a 10 mM solution of sodium formiate in 50% isopropanol that was infused during LC reequilibration using a divert valve equipped with a 20 μL sample loop.

Data were processed with DataAnalysis 4.0 SP5 using an inhouse-VBA-script to dissect compound peaks, determine monoisotopic masses and export datasets allowing access to METLIN and Massbank MS/MS databases and KNApSAcK Species–Metabolite Relationship database. Compounds with monoisotopic masses known for the genus *Lippia* in KNApSAcK database were screened in METLIN and/or Massbank and finally compared to original literature data or interpreted when no suitable literature data were available.

2.7. Determination of antioxidant activity

The antioxidant activity of the extract was determined by spectrophotometric methods according to Kulisic et al. (2004). Solutions of 1 and 4 mg/mL of extracts were prepared. O_2-saturated water was used as oxidant reagent after pumping air in water for 30 min. Tween® 40 (200 mg), β-carotene (0.5 mg), linoleic acid (20 μL) (all reagents from Fluka, Steinheim, Germany) and chloroform (0.5 mL) (Anedra, Argentina) were mixed, the chloroform was evaporated, and the resulting precipitate was suspended in 50 mL of the aerated water.

0.2 mL of the extracts to be tested was mixed in glass tubes with the emulsion (4 mL). The absorbance was immediately determined at λ=470 nm, and then in time intervals of 20 min during an incubation time of 2 h at 55 °C. Negative control: distilled water; positive control: butylhydroxytoluene (BHT) (Fluka, Steinheim, Germany) at different concentrations (1 and 4 mg/mL). The relative antioxidant activity AA [%] was determined by the following equation:

$$AA[\%] = 100 \times (\text{Ab sample}_{120\ \text{min}} - \text{Ab control}_{120\ \text{min}} / \text{Ab control}_{0\ \text{min}} - \text{Ab control}_{120\ \text{min}})$$

2.8. Determination of free radical scavenging activity (DPPH assay)

The free radical scavenging was determined according to Wei and Shibamoto (2007). Dilutions of extracts (1.5 mL) were mixed in glass tubes with 0.5 mL of 2,2-diphenyl-1-picrylhydrazyl DPPH (0.012%), (Sigma, Steinheim, Germany), incubated in the dark for 20 min at room temperature, followed by determination of the absorbance at λ=515 nm. Negative control distilled water; positive control BHT (4 mg/mL). The relative scavenging activity SA [%] was estimated by the following equation:

$$SA\% = 100 \times (\text{Ab control} - \text{Ab sample} / \text{Ab control})$$

2.9. Cell lines, bacteria and cultivation

AGS cells (ATCC CRL-1730), an adherent human gastric adenocarcinoma epithelial cell line, were kindly provided by Prof. Dr. med. Winfried Beil (Medizinische Hochschule Hannover, Germany). Cells were cultured in RPMI 1640 (PAA Laboratories, Cölbe, Germany) supplemented with 10% FCS (PAA Laboratories, Cölbe, Germany) and 1% antibiotics (streptomycin/penicillin, PAA Laboratories, Cölbe, Germany), at 5% CO_2/37 °C. Passaging was performed once a week to a maximum of 20 passages.

Murine macrophage cell line RAW 264.7 (ATCC TIB-71) was cultivated and incubated in DMEM medium (high glucose with glutamine, supplemented with 10% FCS, Thermo Scientific, Waltham, USA) and 1% penicillin 10.000 U/mL/streptomycin 10 mg/mL (PAA Laboratories, Cölbe, Germany) in a humidified incubator at 8% CO_2/37 °C. Sub-cultivation was carried out every second or third day by gentle scraping when the cells had reached a confluence of about 80% (Maas et al., 2011). For functional testing, passages 30–60 were used.

Helicobacter pylori ATCC 700824 (strain J99, identification for quality control by PCR for *vacA*, *cacA* genes) was cultivated for two or three passages to minimize the risk of phase-variable switching of *omp* genes. Cultivation was performed according Niehues et al. (2010).

2.10. Influence of Lippia integrifolia extracts on cellular viability (MTT assay, Mosmann, 1983)

To determine the influence of test compounds on cell viability, AGS cells were cultured in 96-well plates (Translucent, Sarstedt, Nümbrecht, Germany) with 5×10^4 cells/well for 24–48 h at 37 °C. At 80% confluence the medium was discarded and cells were washed twice with PBS (100 μL/well). Test solution (100 μL/well) containing extract of *Lippia integrifolia* (infusion, decoction or its fractions; 1–200 μg/mL) was added, followed by incubation for 24 h. Untreated control cells, cultivated only with fresh RPMI medium, served as negative control, while cells supplemented with 10% FCS were used as positive control. After incubation with extracts, the cell viability was determined by MTT assay. The supernatant medium in the wells was discarded and cells were gently washed twice with 200 μL PBS/well. 50 μL of MTT were added into each well and the plates were incubated for 4 h/37 °C. MTT was removed from the plates and 50 μL DMSO/well were added to dissolve the insoluble formazan crystals. After 5 min the absorbance of the formazan was measured (Tecan Sunrise, Tecan Austria, Salzburg, Austria) at λ=492 nm against reference wavelength λ=690 nm.

2.11. Influence of extracts of Lippia integrifolia on cellular proliferation BrdU incorporation ELISA (Porstman et al., 1985)

AGS cells were cultured for 24 h in 96-well plates. Solutions of the test compounds (100 μL/well) in FCS-free medium were added and cells were incubated for another 24 h at 37 °C. Cellular proliferation was performed by BrdU cell proliferation kit (Roche Diagnostics, Mannheim, Germany) according to instructions of the manufacturer. The resulting color was measured with a Tecan Sunrise (Tecan Austria, Salzburg, Austria) at λ=450 nm against reference wavelength λ=690 nm.

2.12. Phagocytosis of FITC-labeled zymosan particles (Maas et al., 2011)

Zymosan A particles, 1 mg (from *Saccharomyces cerevisiae*, Sigma, St. Louis, USA) were suspended in 1 mL of carbonate buffer, pH 9.6. 10 μL of FITC solution (fluorescein isothiocyanate, ICN Biomedicals, Irvine, USA; 10 mg/mL in DMSO) was added and the mixture was incubated for 1 h at room temperature in the dark. The labeled particles were washed thrice with PBS. RAW 264.7-macrophages were seeded in black 96 well plates at a

density of 1×10^5 cells per well. The cells were incubated for 2 h to ensure adherence. *Lippia integrifolia* extracts or LPS 1 μg/mL (from *Escherichia coli* 055:B5, Sigma-Aldrich, St. Louis, USA) were added and the incubation continued for 4 h. The medium was replaced by zymosan suspension (5×10^6 particles per well, cell to particle ratio 1:50) and phagocytosis was allowed to take place for 2 h. The suspension was removed by vacuum aspiration and 100 μL of trypan blue solution (4 mg/mL, diluted 1:16 with PBS, Fluka, Buchs, Switzerland) were added in order to quench fluorescence of adhering, but not ingested particles. After 1 min of incubation at room temperature, trypan blue was removed, and the fluorescence of ingested particles was measured by Fluoroskan Ascent FL (Thermo Scientific, Waltham, USA) at excitation wavelength of $\lambda = 485$ nm and a measuring wavelength of $\lambda = 538$ nm.

2.13. Influence of aqueous extracts of Lippia integrifolia on NO-release (Maas et al., 2011)

RAW 264.7 macrophages were seeded in 96 well plates at a density of 1×10^5 cells per well in the presence of test compounds. For the test on inhibition of LPS-stimulated NO production, cells were incubated with LPS 1 μg/mL alone or co-incubated with test extracts and LPS 1 μg/mL for 24 h. Aminoguanidine 100 μM plus LPS 1 μg/mL served as a control for the reduction of NO production (Koh et al., 2009), NO production was determined by Griess reagent (1% sulfanilamide, 0.1% naphthyl-ethylenediamine-dihydrochloride, 4.3% H_3PO_4; Fluka, Buchs, Switzerland) by mixing 50 μL of cell culture supernatant with the same volume of reagent. After incubation for 10 min at room temperature, the absorption was measured at $\lambda = 540$ nm against $\lambda = 690$ nm (Sunrise microplate reader, Tecan, Grödig, Austria). The nitrite concentration was determined by comparison with a sodium nitrite standard calibration curve in culture medium (5–100 μM).

2.14. Agar diffusion test

To exclude unspecific cell toxicity of test compounds against *Helicobacter pylori* a disk diffusion test was performed at different concentrations between 100 and 2000 μg/mL of test compounds using BD Sensi-Disks (Becton and Dickinson, Heidelberg, Germany), placed on agar plates; positive control amoxicillin (0.5 μg per disk, MP Biomedicals, Irvine, USA). The incubation time was between 3 and 4 days under microaerophilic condition (CampyGen Container System - Gaspak, Oxoid, Ltd., UK).

2.15. Antiadhesive effect of aqueous extract of Lippia integrifolia against Helicobacter pylori on AGS cells (Niehues et al., 2011)

The assay was performed according to the methods described by Niehues et al. (2011). *Helicobacter pylori* was inoculated on Tryptic Soy Agar - Difco™ (TSA) plates (Merck, Darmstadt), supplemented with 5% defibrinated sheep blood (Oxoid, Ltd. Wesel) and incubated at 37 °C under anaerobic conditions for 48 h. AGS cells were seeded with 200,000 cells/well and grown for 48 h to a confluence of 80%.

Helicobacter pylori was pre-incubated with different concentrations of *Lippia integrifolia* extracts in RPMI medium (0.5, 1, 2 mg/mL) at 37 °C in a shaker. Bacteria were collected by centrifugation (5000*g*, 5 min), washed twice with PBS and resuspended in fresh RPMI fresh medium (2 mL). Afterwards, the bacteria suspension was added to AGS cells grown to 80% confluence in 6 well plates and incubated for 1 h at 37 °C/5% CO_2. Samples were analyzed by flow cytometry (FACS-Calibur, BD, Heidelberg, Germany).

2.16. Cytokine secretion of AGS cells after Helicobacter pylori infection

AGS cells were inoculated in 6 well plates at a cell density of 2×10^4 per well and incubated at 37 °C in CO_2 atmosphere for 48 h (confluence approx. 80%). *Helicobacter pylori* in TSA plates were harvested and a bacterial suspension was diluted in fresh RPMI to an OD of 0.05 in each well, with a final volume of 2 mL. The multiplicity of infection (MOI) was 1:150, referred to AGS:*Helicobacter pylori* ratio. For investigation of the influence of *Lippia integrifolia* extracts on *Helicobacter pylori* infected AGS cells solutions of the test compound (1–1000 μg/mL) were added to the wells. After 24 h of incubation time, the media were removed by centrifugation at 5283*g*, 5 min and stored at −20 °C for further analysis. IL-6, IL-8 and TNF-α were determined in these samples by ELISA kit (PeproTech, Hamburg Germany) according the instructions of the manufacturer.

2.17. Statistical analysis

The analysis was performed by using SPSS®. The experimental results are expressed as the mean ± SD. Data were assessed by analysis of variance. In case the analysis indicated significant differences between groups, each group was compared by Dunnett's *t*-test (two-sided) and $p < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Phytochemical characterization

From the aerial parts of *Lippia intergrifolia* two aqueous extracts, infusion and decoction, were prepared. After lyophilization yields of 3.6% and 3.7%, respectively, were determined. Flavonoid content was determined with 56.1 ± 1.4 mg/g dry extract for the infusion and with 50.6 ± 1.6 mg/g dry extract for the decoction, calculated as quercetine. Total phenolic content was determined with 13.00 ± 0.26 mg/g dry extract for the infusion and with 11.10 ± 0.13 mg/g dry extract for the decoction, calculated as gallic acid.

LC−MS profiling of the decoction extract indicated the presence of flavonoids, phenylethanoids, secoiridoid glycosides and sesquiterpenes as main constituents of the decoction, according to TIC-peak intensity (Fig. 1 and Table 1). Flavonoids were tentatively identified by their UV− and mass spectra as luteolin hexosides bearing an additional hydroxyl group (**1**, **3**) or methoxy group (**2**, **4**, **8**) at their A-ring (Cuyckens and Claeys, 2004). Also, methoxylated apigenin hexosides were found (**5**, **9**, **15**), a dihydrochalcone (**6**) with a fragmentation pattern strongly resembling to phloretin-3′,5′-di-C-glucoside (Barreca et al., 2011; De Beer et al., 2012) and a free flavone agylcon matching salvigenin (**19**). These data match the presence of salvigenin, 6-hydroxyluteolin, 6-methylscutellarein and phloretin derivatives in other *Lippia species*, at least with regard to the presence of their respective aglyca (Ono et al., 2006, Lin et al., 2007, Funari et al., 2012). Two further bismethoxylated flavone hexosides were found, each characterized by the loss of methane from its respective aglycon during CID (**13**, **14**). This behavior is characteristic for flavonoids bearing two methoxy groups in the B-ring (Li et al., 2012). Acteoside (**7**), isoacteoside (**10**) and dimethyl-secologanoside (**11**) have been described for the genus *Lippia* (Catalán and de Lampasona, 2002; Funari et al., 2012) and were identified in the decoction extract by their respective UV spectra, MS/MS signals and − in cases of **7** and **10** − their retention order (Liu et al., 2011). The main sesquiterpenes that were detected by LC−MS were

tentatively identified as (Epi)lippidulcine A (**16**, **17**) and their oxidation products (**18**, **20**) with modifications in the side chain. Though no MS/MS data were available for these compounds as a reference, the observed fragments at *m*/*z* 111 and 125 are indicative for a retro-aldol-condensation of their β-hydroxyketone structure. **18** and **20** show an additional fragment at *m*/*z* 141, indicative for an additional oxygen atom at their side chain. Ono et al., 2006 described the presence of peroxylippidulcines with the respective structures.

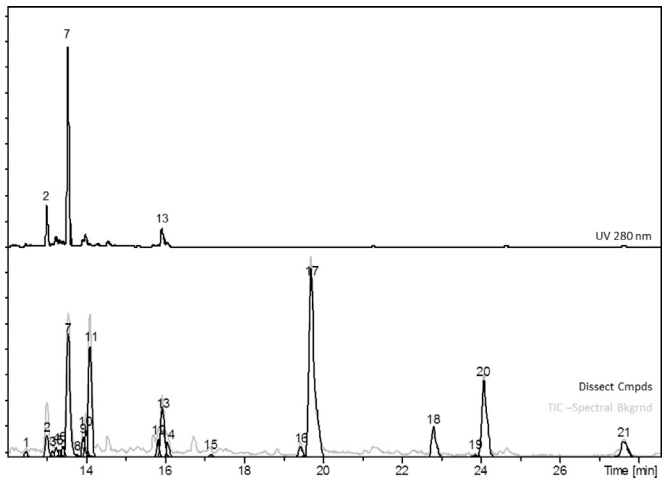

Fig. 1. Fingerprint chromatograms of the decoction extract from dried leaves of *Lippia integrifolia*. Dissect compounds overlayed with the TIC represent the elution profiles of tentatively identified constituents.

During the phytochemical investigations the presence of a quite high polysaccharide content got obvious. For that raw polysaccharides (RPS) were isolated by ethanol-precipitation from the decoction extract in yields of about 9%. Additionally, polysaccharide depleted, non-ethanol precipitable material was isolated additionally and named in the following as phenolic fraction PhF.

TFA hydrolysis of RPS and quantitation of the carbohydrate monomers indicated the presence of arabinose (38%) and galactose (21%) as main building blocks, beside glucose (18%), rhamnose (13%), mannose (5%) and xylose (5%), pinpointing to the existence of an arabinogalactan.

LC–MS profiling of PhF resulted in qualitatively more or less the same peak pattern compared to the chromatograms obtained from the decoction extract (Fig. 1). Some minor quantitative shifts got obvious when calculating the ratio of particular peaks found in the decoction extract and in PhF (Table 1). For most of the identified compounds in PhF about 30% higher concentrations were calculated in comparison to the decoction extract, which is explainable by the removal of about 30% of polysaccharides during the fractionation procedure. For peak 6 (Phloretin-di-C-hexoside) in PhF a strong reduction in content was observed, and also peaks 18 and 20, corresponding to Peroxylippidulcines, decreased

Table 1
Peak assignments and analytical data of the aqueous decoction extract from dried leaves of *Lippia integrifolia* according LC-MS/DAD analysis. Ratio was calculated from the peak areas of EICs for $[M+H]^+$ or $[M+Na]^+$.

Compound no.	t_R/ min	*m*/*z*	Sum formula	λ_{max}/nm	Tentative identification	Ratio decoction/ PhF
1	12.51	465.0968 $[M+H]^+$, 303.0472, 135.0436, 123.0081	$C_{21}H_{20}O_{12}$	282, 344	6-Hydroxyluteolin-hexoside	1.7
2	13.05	479.1111 $[M+H]^+$, 317.0598, 302.0374, 168.0036	$C_{22}H_{22}O_{12}$	255, 272, 345	6-Methoxyluteolin-O-hexoside (Nepetin-O-hexoside)	1.1
3	13.19	465.0945 $[M+H]^+$, 303.0455, 169.0102, 123.0047	$C_{21}H_{20}O_{12}$	253, 269, 344	6-Hydroxyluteolin-hexoside	0.8
4	13.27	479.1101 $[M+H]^+$, 317.0636, 302.0373, 168.0026	$C_{22}H_{22}O_{12}$	281, 335	6-Methoxyluteolin-hexoside	1.3
5	13.36	463.1172 $[M+H]^+$, 301.0680, 286.0434, 168.0007	$C_{22}H_{22}O_{11}$	283, 327	6-Methylscutellarein-hexoside	1.4
6	13.45	599.1841 $[M+H]^+$, 581.1759, 563.1674, 545.1597, 527.1288, 479.1443, 461.1328, 449.1157, 431.1175, 419.1398, 107.0474+	$C_{27}H_{34}O_{15}$	230, 285, 364	Phloretin 3′,5′-di-C-hexoside	0.2
7[a]	13.57	625.2053 $[M+H]^+$, 479.1486, 471.1429, 325.0871, 163.0373	$C_{29}H_{36}O_{15}$	246s, 292s, 330	**Acteoside**	**0.5**
8	13.80	479.1149 $[M+H]^+$, 317.0555, 302.0377, 168.0112	$C_{22}H_{22}O_{12}$		6-Methoxyluteolin-hexoside	1.4
9	13.96	463.1198 $[M+H]^+$, 301.0646, 286.0458, 168.0039	$C_{22}H_{22}O_{11}$	228, 272, 350	6-Methoxyscutellarein-hexoside	1.3
10	14.02	625.2053 $[M+H]^+$, 479.1492, 471.1417, 325.0898, 163.0373	$C_{29}H_{36}O_{15}$	246s, 292s, 330	**Isoacteoside**	**7.7**
11	14.13	419.1503 $[M+H]^+$, 257.0969, 239.0934, 225.0703, 207.0666, 193.0398, 165.0517, 151.0342, 123.0393	$C_{18}H_{26}O_{11}$	235	Dimethyl-secologanoside	1.1
12	15.84	485.1630 $[M+H]^+$, 339.1039, 177.0534, 117.0318	$C_{22}H_{28}O_{12}$		Unknown phenolic diglycoside (prob. Ferulic acid di-hexoside)	0.53
13	15.95	493.1299 $[M+H]^+$, 331.0786, 315.0531, 298.0455, 270.0492, 242.0499, 136.0166, 108.0209	$C_{23}H_{24}O_{12}$	242, 275, 336	B-ring-dimethoxylated Flavone-hexoside	1.3
14	16.09	477.1340 $[M+H]^+$, 315.0813, 299.0507, 282.0492, 254.0351, 226.0559, 136.0160, 108.0212	$C_{23}H_{24}O_{11}$	277, 324	B-ring-dimethoxylated Flavone-hexoside	1.2
15	17.17	463.1203 $[M+H]^+$, 301.0693, 286.0393, 258.0518	$C_{22}H_{22}O_{11}$	248, 268, 337	Methoxylated apigenin-hexoside	1.4
16[a]	19.43	253.1760 $[M+H]^+$, 235.1689, 125.0955, 111.0809, 93.0695	$C_{15}H_{24}O_3$		(Epi)Lippidulcine A	1.6
17[a]	19.69	253.1779 $[M+H]^+$, 235.1671, 125.0959, 111.0807, 93.0701	$C_{15}H_{24}O_3$	226	(Epi)Lippidulcine A	1.1
18[a]	22.77	291.1556 $[M+Na]^+$, 251.1650, 141.0911, 125.0961, 111.0811, 93.0694	$C_{15}H_{24}O_4$		Peroxylippidulcine	**0.03**
19[a]	23.82	329.1003 $[M+H]^+$	$C_{18}H_{16}O_6$	214, 276, 334	Salvigenin	2.4
20[a]	24.04	291.1557 $[M+Na]^+$, 251.1632 141.0920, 125.0971, 111.0814, 93.0705	$C_{15}H_{24}O_4$		Peroxylippidulcine	**0.01**
21	27.56	235.1706 $[M+H]^+$, 217.1592, 137.0975, 111.0803, 91.0535	$C_{15}H_{22}O_2$		Unknown Sesquiterpene	0.4

[a] No suitable precursor ion was available for these compounds, MS/MS data were taken from Dissect spectra. Tentative identification was achieved by comparison with available literature MS/MS data or *de novo* interpretation of MS/MS spectra. See the text for references and further details.

strongly, probably due to the destruction of the labile peroxide structure. On the other side a strong increase in isoactoside (peak 10) was observed in PhF, while peak 7, corresponding to acteoside strongly decreased. This shift is explainable by an intramolecular transesterification of the caffeic acid moiety from O6 to O4 of the glucose residue. As the intensity ratio of [M+H] to [M+Na] for acteoside and isoacteoside increases with concentration, the peak areas obtained from EICs of m/z 625 $[M+H]^+$ are biased. Analysis of unbiased UV peak areas indicated that the sum of acteosid and isoacteoside was 20% higher in PhF which is comparable to other phenolics. About 55% of the acteoside present in the decoction rearranged to isoacteoside during preparation of PhF.

From these investigations fraction PhF is assessed to have qualitatively the same composition as the decoction extract, with quantitatively containing a higher concentration of phenolic compounds.

3.2. Extracts influence bacterial adhesion of Helicobacter pylori to human stomach cells

Neither infusion, decoction, RPS or PhF (100–2000 µg/mL) exerted any cytotoxic effects within agar diffusion test against *Helicobacter pylori* (data not shown).

For investigation of a potential inhibition of bacterial adhesion of *Helicobacter pylori* to stomach host cells an *in vitro* flow cytometric assay with human gastric epithelial AGS cells and FITC-labeled bacteria was used to quantify potential antiadhesive effects (Messing et al., 2014). Antiadhesive effects of about 20 to 60% were found in cases of *Helicobacter pylori* were pretreated for 2 h with the infusion, decoction extract, RPS and PhF (Fig. 2). Between 500 and 2000 µg/mL no clear concentration dependence was detected for the both extracts and RPS. In contrast to that PhF inhibited bacterial adhesion up to 60% in a dose-dependent manner.

Using a different incubation protocol with a 2 h preincubation of AGS cells with the extracts and fractions, followed by removal of the test fractions and adding the FITC-labeled bacteria to the eukaryotic cells no inhibitory effects were measured (data not shown). This clearly indicates that the *Lippia integrifolia* compounds act against the bacterial adhesions and have no affinity against the complementary molecular targets of the eukaryotic host cells.

It has to be realized that quite high doses of extract or PhF had to be used for inhibition of bacterial adhesion. This is in accordance to the literature published on other antiadhesive candidates against *Helicobacter pylori*, using for example gram-doses for the clinical testing of sialyllactose against *Helicobacter pylori* (Parente et al., 2003).

From these data it can be deduced that *Lippia integrifolila* extract might influence *Helicobacter pylori* adhesion to stomach tissue, and therefore influence gastric inflammation at a very early point of bacterial infection. This could rationalize the traditional use of herbal tea preparation from this species for gastric inflammation.

3.3. Extracts and fractions stimulate cell viability of stomach cells, but do not induce cellular proliferation

To get more insight into a potential gastroprotective activity of the extracts the respective influence on the cell physiology of human stomach cells (AGS cell line) was investigated under *in vitro* conditions. Infusion, decoction RPS and PhF stimulated cellular vitality of AGS cells significantly at concentrations >10 µg/mL as determined by quantification of succinate dehydrogenase as marker for mitochondrial activity by MTT assay (Fig. 3A). Interestingly this increased mitochondrial activity did not lead to an increased cellular proliferation (Fig. 3B) as determined by BrdU incorporation ELISA.

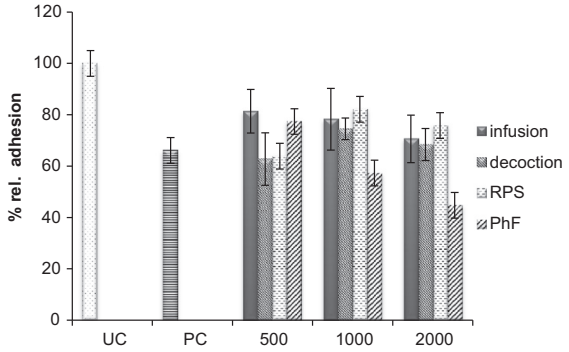

Fig. 2. Relative adhesion (%, related to the untreated control UC) of FITC-labeled *Helicobacter pylori* to AGS cells after 2 h pretreatment of the bacteria with different concentrations of *Lippia integrifolia* extracts (infusion, decoction). Negative control, untreated bacteria: UC; positive control: PC, Okra fresh extract (Messing et al., 2014). Values are mean ± SD; $n=3$ independent experiments with 3 replicates each.

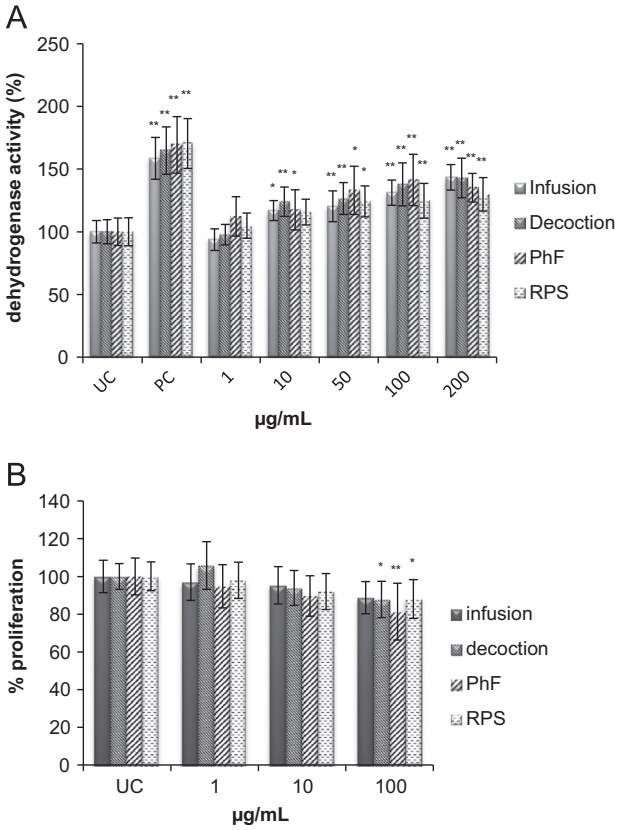

Fig. 3. Influence of aqueous extracts, RPS and PhF from *Lippia integrifolia* on cellular viability by MTT assay (A) and proliferation rate by BrdU-ELISA (B) of human stomach AGS cells, incubated for 24 h with test compounds (1–200 µg/mL). Bars represent standard deviation (SD) with $^{*}p<0.05$, $^{**}p<0.01$ compared to the untreated control group (UC); PC: positive control, 10% FCS supplementation to media.

3.4. Extracts from Lippia integrifolia decrease Helicobacter pylori induced inflammation

To investigate if this higher cellular viability can lead to a changed inflammatory or anti-inflammatory response against *Helicobacter pylori* infection AGS cells were incubated with infusion and decoction

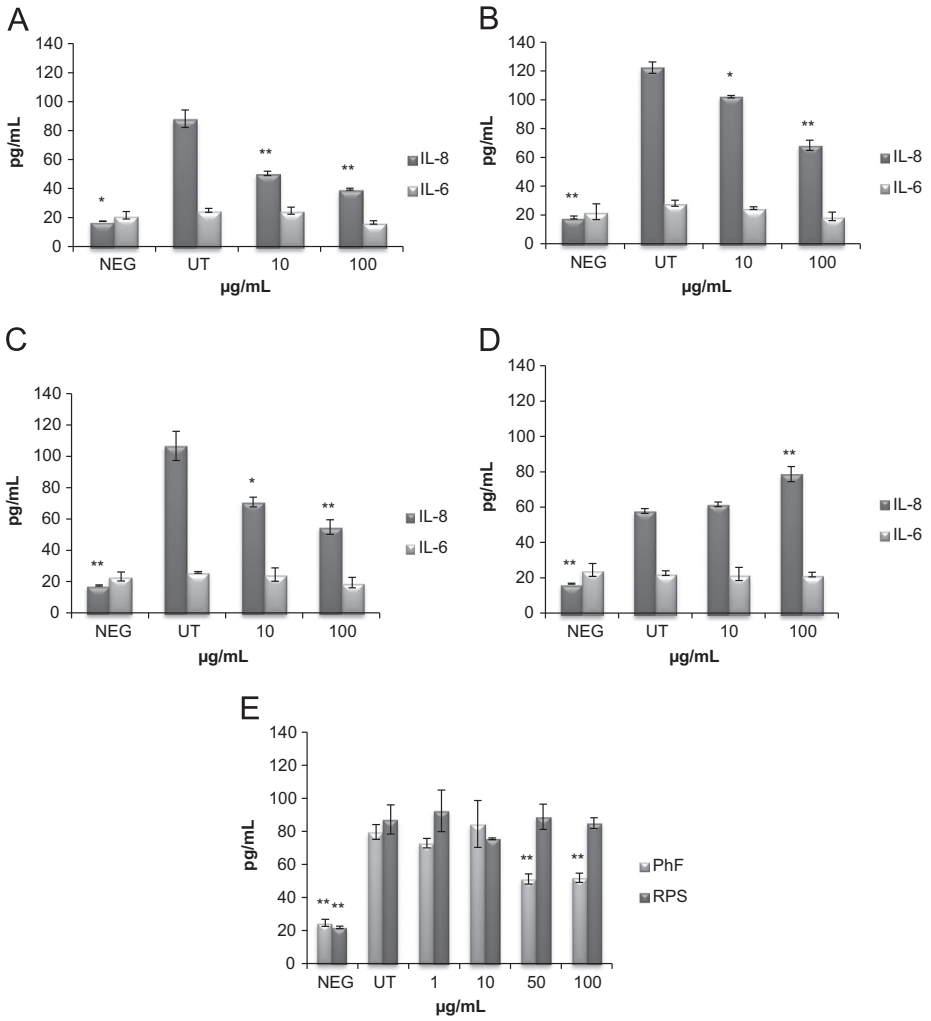

Fig. 4. Secretion of Il-6 and IL-8 by AGS cells after co-incubation of AGS cells with *Helicobacter pylori* (MOI 1:150) in the presence of different concentrations (10, 100 µg/mL) of infusion (A), decoction (B) for 24 h. Additionally preincubation with and without washing steps after incubation were made. C: after 6 h of pre-incubation with infusion, FITC-labeled *Helicobacter pylori* was added and then incubation until 24 h. D: after 6 h incubation with infusion, the medium was removed and the cells were washed with PBS, then *Helicobacter pylori* were added and incubated until 24 h. E: the sub-fractions RPS and PhF were tested only for IL-8 (24 h of co-incubation). NEG: negative control, AGS cells not infected with bacteria, no *Lippia integrifolia* extract treatment; UT: inflammation control, AGS cells, infected with bacteria, no *Lippia integrifolia* extract treatment. Significances are calculated against respective values of UT control group, $^{*}p < 0.05$, $^{**}p < 0.01$.

and bacterial infection was triggered by subsequent addition of *Helicobacter pylori* (multiplicity of infection 1:150). IL-6 and IL-8 were quantitated as typical inflammation maker (Fig. 4).

In a first set of experiments AGS cells were co-incubated with *Helicobacter pylori* for 24 h in the presence of infusion (Fig. 4A) or decoction extract (Fig. 4B). IL-8 titers in the untreated control groups (*Helicobacter pylori* infected, no treatment with extract) increased about 5–6 fold after bacterial infection. Addition of infusion extract (Fig. 4A) and decoction extract (Fig. 4B) led to a significant reduction of IL-8 titers after bacterial infection. Interestingly no changes were observed for IL-6 secretion after *Helicobacter pylori* infection of the AGS cells, indicating that this cytokine is not a key player for that pathogen in this cell line.

Investigation of the subfractions RPS and PhF in this coincubation protocol indicated significant inhibition of IL-8 release by PhF, but not by the polysaccharide fraction (Fig. 4E). PhF (non-ethanol precipitable, low molecular material) at >50 µg/mL significantly inhibited the infection-induced IL-8 release, and therefore might be considered as active fraction of the herbal material. This means that probably the phenolic or flavonoid content of this PhF fraction contributes mainly to a potential anti-inflammatory effect in stomach cells. Special focus should be devoted within follow-up investigations to high amounts of acteosid and isoactoside, compounds to be known as anti-inflammatory agents (He et al., 2011)

In the next experiments the incubation protocol was varied: AGS cells were preincubated with infusion extract for 6 h, then *Helicobacter pylori* were added – still in the presence of extract – and incubation was performed until 24 h in total (Fig. 4C). In principle again significant lower IL-8 titers were found in the extract-treated groups compared to those in the untreated control.

In a next set of similar experiments AGS cells were again preincubated with infusion extract for 6 h, followed by removal of the extract from the incubation assay. After this procedure *Helicobacter pylori* was added and incubation performed until 24 h in total (Fig. 4D). No changes on IL-6 and IL-8 were observed in this setup, indicating that the extract compounds have to be present during the infection phase for potential anti-inflammatory effects. Cytoprotective effects of the extract against host cells can be excluded by this experiment.

3.5. Extracts reduce inflammatory response in RAW macrophages

Potential anti-inflammatory effects were analyzed in a cell-based *in vitro* assay using murine RAW 264.7 macrophages and NO

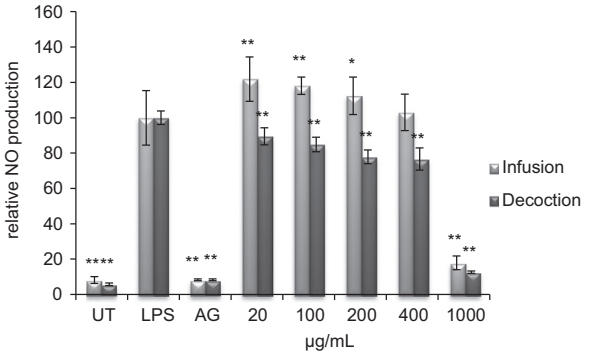

Fig. 5. Influence of infusion and decoction of *Lippia integrifolia* on NO production (determined as nitrite via Griess reaction) of RAW 264.7 macrophages after coincubation with LPS (1 μg/mL) and extracts at different concentrations for 24 h. Three independent experiments with $n=6$, mean ± standard deviation. *t*-test against LPS: $^{*}p<0.05$, $^{**}p<0.01$. UT=untreated control; PC: LPS (1 μg/mL) stimulated control; AG=reduction control, aminoguanidin 100 μM.

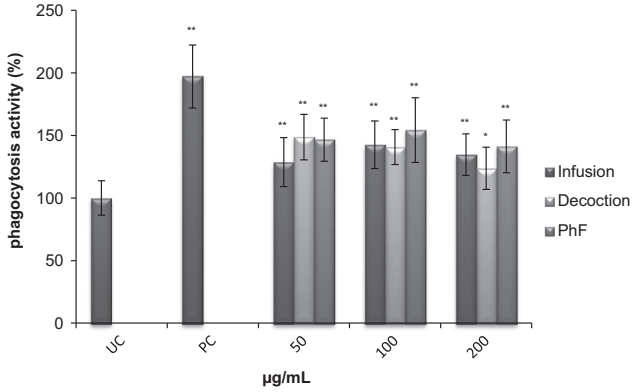

Fig. 6. Relative phagocytosis activity of murine macrophages RAW 264.7 against FITC-labeled Zymosan particles for determination of influence of aqueous extracts of *Lippia integrifolia* on phagocytosis activity RAW 264.7 were incubated at concentrations of 50 to 200 μg/mL for 4 h. UC: untreated cells; PC: Positive control after stimulation with LPS (1 μg/mL). Bars represent standard deviation (SD) with $^{*}p<0.05$, $^{**}p<0.01$ compared to the untreated control group (UC).

Table 2
Radical scavenging and antioxidative activity of infusion and decoction extract from *Lippia integrifolia*. Scavenging activity refers as ppm, the concentration corresponds to IC_{50}; Antioxidative activity refers as percentage (%) related to BHT with all sample solutions prepared at 4000 ppm. All experiments were performed as 3 independent replicates. BHT served as positive control.

Sample	Radical scavenging activity (ppm, IC50)	Antioxidant activity (%) at 4000 ppm
Infusion	13.8 ± 4.2	43.4 ± 8.7
Decoction	18.8 ± 5.6	58.4 ± 9.6
BHT	19.3 ± 7.8	96.7 ± 5.4

formation as central parameter for initiation of the inflammation cascade. As shown in Fig. 5 untreated macrophages showed nearly no NO formation; LPS-stimulated cells (1 μg/mL) were used as positive control and the secreted NO amount was set as 100%. Aminoguanidin was used as inhibition control for NO-synthase (Koh et al., 2009). Incubation of the LPS-stimulated cells with the decoction extract for 24 h resulted in a dose-dependent (20–400 μg/mL) decrease of NO-formation, indicating potential anti-inflammatory effects. Interestingly, incubation with the infusion did not lead to such inhibitory effects. Very low NO formation (< 10%) was observed at 1000 μg/mL extract concentration, but this is assessed to be due to unspecific cell toxic effects.

3.6. Extracts stimulate phagocytosis rate in RAW macrophages

A further characteristic of macrophages is their ability for phagocytosis, which again can be associated with an increased innate immune defense. Using again the RAW 264.7 macrophages infusion, decoction and PhF fraction increased significantly phagocytosis rates against fluorescent-labeled zymosan particles for about 40–50% in the concentration range from 50 to 200 μg/mL (Fig. 6).

3.7. Extracts show radical scavenging activity

Modulation of anti-inflammatory responses may be modulated by reduction of oxidative stress. For investigations of *in vitro* anti-oxidative capacity of *Lippia integrifolia* extracts were tested concerning antioxidant activity by quantitation of oxidized carotene products against BHT as positive control (Kulisic et al., 2004) and by free radical scavenging activity in DPPH assay against BHT as positive control. As shown in Table 2 anti-oxidative activity of infusion and decoction at 4000 ppm was lower compared to that of BHT while radical scavenging activity for both extracts had been comparable to that of BHT. According to these data strong *in vitro* radical scavenging activity is obvious, but only further *in vivo* studies can prove that the relevant secondary compounds will indeed get absorbed into the systemic compartment after oral application of *Lippia integrifolia* extract.

4. Conclusion

Summarizing the traditional use of aqueous extracts from *Lippia integrifolia* for gastric inflammation seems to be rationalized by these investigations: besides anti-inflammatory effects on stomach cells antiadhesive properties of the extracts against the main bacterial inductor of gastritis *Helicobacter pylori* were identified. Additional effects for stimulation of innate immunity and potential radical scavenging effects may additionally contribute to the activity of the extracts. From this point of view the traditional use of *Lippia integrifolia* extracts seems to be justified by the *in vitro* data, but further clinical studies should be performed to verify the use of *Lippia* extracts for efficient gastroprotection.

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