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In vitro activity of dried extracts of *Nepeta cataria* against *Helicobacter pylori* strains

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Abstract

Nepeta cataria L. (Lamiaceae) is widely used in popular medicine for the treatment of several gastrointestinal disorders. Drying procedures were applied to infusions prepared at 10% and 15% (w/v) from dried grounded aerial parts of *Nepeta cataria*. Freeze-drying extracts (FDEs) and spray-dried extracts (SDEs) were obtained. The activity against *H. pylori* of SDEs and FDEs was evaluated. FDEs were active against *H. pylori* in the three strains evaluated at 10 and 0.31 mg/ml. SDEs were not active against *H. pylori* at any concentration.

Chemical fingerprints were achieved by Capillary Electrophoresis (CE). The infusion, SDE and FDE profiles showed a similar chemical composition. However, at 6.1 min, a peak with analogous corrected area was detected in the infusion and FDE profiles; in addition it was not found in the SDE. In the FDE samples the main components concentration was lower than in the infusion and SDE samples. The highest concentration of the metabolites was found in the SDE samples.

Minor components of the infusion that are not present after the spray drying procedure can be related with the antibacterial activity. Further research aimed the identification and purification of anti-*H. pylori* components from the *N. cataria* are necessary as well as to determine the degree of toxicity of these extracts.

Key words: Nepeta cataria, Helicobacter pylori, dried extracts, capillary electrophoresis

Introduction

There are several reports about the use of *Nepeta cataria L.* (Lamiaceae) for the treatment of gastrointestinal disorders in folk medicine (2,12,24) *N. cataria* has been traditionally used for the treatment of colds, colic, fevers and gynecological disorders (17). Also, it has been employed for its carminative, antispasmodic and sedative properties in popular medicine (1).

There are a limited number of studies about its biological activities, including antibacterial, antifungal, analgesic and behavioral (7). In addition, its insect repellent action has been reported (5, 19).

Nepetalactone compounds mainly found in the essential oils of the *Nepeta* species are considered to be responsible for the pharmacological properties (16). The activity of the essential oil and nepetalactones of two *Nepeta* species against *Helicobacter pylori* has been reported (11). However, the inhibitory effects of its aqueous extracts against *H. pylori* have not been extensively studied considering that this herb is traditionally consumed in tea form.

H. pylori colonize the human stomach where it can induce peptic ulcers, mucosa-associated lymphoid tissue (MALT) lymphoma, and gastric adenocarcinoma (8, 18). The regime for the eradication of *H. pylori* infection currently includes a triple therapy, which combines the antibiotic clarithromycin (CLA) or metronidazole (MTZ) and amoxicillin (AMX) with a proton pump inhibitor such as omeprazole (OMZ). This chemotherapy produces side-effects and fails to eliminate infection in 10–30% of patients (3). The prevalence of strains resistant to CLA and MTZ in our region is high compared to other region of world (22); therefore, it is nowadays important to search for substances with anti-*H. pylori* activity. Medicinal plants have been used as traditional remedies in treating and preventing gastrointestinal diseases for hundreds of years, and their anti-*H. pylori* activity has been widely demonstrated in vitro (20, 21, 23).

Spray drying and freeze-drying are well-established and widely used techniques to turn liquid into solid forms since the dried extracts present several pharmaceutical advantages over the conventional liquid form (6). In addition, spray-drying is the most commonly used method in the herbal processing industries. The resulting products exhibit improved technological properties as well as lower storage costs and higher concentration and stability of the active substances (13, 14).

With the goal of finding out more extensively the traditional uses of *N. cataria* aqueous extracts for the treatment of gastrointestinal disorders, the in vitro activity against *H. pylori* strains was carried out. The achievement of more stable solids from the aqueous extracts (teas) was proposed with the aim of designing phytopharmaceutical dosage forms with improved technological properties. Thus, spray drying and freeze-drying procedures were applied to *N. cataria* aqueous extracts. Also, the activity of the obtained products (freeze-drying extracts, FDE and spray-dried extracts, SDE) and the effect of the applied drying procedures against *H. pylori* were evaluated.

Material and Methods

Plant material

The plant material consisted in dried grounded aerial parts of *N. cataria*, purchased from a local herbal shop (San Luis, Argentina). The plant identity was confirmed by a botanical evaluation according to the description in the Physician's Desk Reference for Herbal Medicines' monography (17) and compared with a voucher specimen maintained in the Herbarium of the Universidad Nacional de San Luis.

Preparation of aqueous extracts

The aerial parts of *N. cataria* were stored in a dark, hermetic recipient until use. Infusions were carried out in the following way: boiling demineralized water (1000 ml) was poured out on 100 and 150 g of ground material and after were left to draw for 20 min. The obtained infusions (10 and 15 % w/v, respectively) were filtered through paper

(Whatman N° 1), the residues were washed with warm water and the volumes were made up to 1000 ml. The clarified infusions were used for both drying processes.

Drying processes

Infusions of two concentration levels (10 and 15% w/v) were subject to two different drying methods: spray drying and freeze-drying.

SDEs were prepared using a BÜCHI 290 Mini-spray Dryer (Büchi Labortechnik AG, Switzerland) with two component nozzle and co-current flow under the following operating conditions: pump setting 10% (3.2 ml min^{-1}), airflow rate of 670 Nl h^{-1} (5 mm) and aspirator rate between 50-100%. The inlet temperature was set at 140°C±2 and 100°C±2 while the outlet temperature was set at 83°C ± 2 and 75°C ± 2, respectively. In the preliminary study, the process parameters (inlet air temperature, aspiration rate, pump rate, and solid concentration) were studied by single factor design.

Infusions were spray-dried in the presence of 15 and 30% (w/w) of colloidal silicon dioxide (Aerosil™); 15% (w/w) of dibasic calcium phosphate (Emcopress™); 15% (w/w) of fumed silica (CAB-O-SIL™) and 30% (w/w) of maltodextrin. Considering the product yielding and the solid appearance, Aerosil™ was selected as a carrier in a concentration of 30 % (w/w) respect to the infusion solid residue.

For FDEs preparation, the infusions were placed on stainless steel trays and freeze-dried using a lyophilizer (Rificor S.A., Argentina) at -70 °C at 0.025 mmHg of pressure. The temperature conditions were: condensate temperature -45°C, initial product temperature -35°C.

Rheological analysis

The obtained solids were subject to a rheological analysis with the aim of selecting the dried extracts with the most suitable technological properties. A LEO 1450 VP scanning electron microscope (SEM) from the Laboratorio de Microscopía Electrónica y Microanálisis (LABMEM) of the Universidad Nacional de San Luis (UNSL) was used to investigate the characteristics of the dried samples.

Chemical fingerprinting analysis

Stock solutions of the SDEs and FDEs were prepared dissolving with water up to a concentration of 10% (w/v), sonicated for 30 min, followed by centrifugation for 10 min and then filtrated through a 0.45 mm Titan Syringe filter (Sri Inc., Eaton Town, NJ. USA). Before electrophoretic analysis, 20 ml and 50 ml of the SDE and FDE stock solutions, respectively, were diluted up to 500 ml with ultrapure water.

The water used in all studies was ultra-high-quality, obtained from a Barnstead Easy pure Reservoir Feed compact ultrapure water system (Dubuque, USA). All solutions were degassed by ultrasonication (Testlab, Argentina).

Chemical fingerprints were obtained by capillary electrophoresis (CE) in a Beckman P/ACE MDQ instrument (Beckman Instruments, Inc. Fullerton, CA) equipped with a diode array detector and a data handling system comprising an IBM personal computer and P/ACE System MDQ Software. Detection was performed at 198 and 203 nm. The fused-silica capillaries were obtained from MicroSolv Technology Corporation (NJ, USA) and had the following dimensions: 60 cm total length, 50 cm effective length, 75 mm ID, 375 mm OD. The temperature of the capillary was kept at 25°C during experiences, and the samples were stored at 25°C. The fingerprints were performed by using a background electrolyte (BGE) composed by sodium tetraborate 20 mM, pH 9.2, 25 kV applied voltage; hydrodynamic mode sample injection, 0.5 Psi during 5 s. Sodium tetraborate ($Na_2B_4O_7.10\ H_2O$) was acquired from Mallinckrodt (Saint Louis, USA). All other reagents and solvents were of analytical grade quality.

Chemical fingerprints of the infusion 10% (w/v) without any drying process was carried out with the aim of compare the major compounds against the

SDE and FDE. Before analysis the infusion was suitable diluted with ultrapure water.

Antibacterial activity

Bacterial strains

For this study, the reference strain of *H. pylori* (NCTC 11638) was obtained from the Microbiology Service of the Hospital Universitario de la Princesa, Madrid, Spain; also, two clinical isolates obtained from gastric antral biopsy specimens were used (HP796 and HP857). *H. pylori* strains were grown in Mueller–Hinton agar supplemented with 7% horse blood under a microaerophilic atmosphere (5% O_2, 10% CO_2, and 85% N_2) at 37 °C for 7–10 days and the identification was based on microaerophilic growth requirement, morphology, Gram's stain, and oxidase, catalase and urease reactions. Strains were stored in trypticase soy broth supplemented with 20% glycerol at –'2d80 °C until use.

Determination of minimal inhibitory concentration (MIC)

The minimal inhibitory concentration (MIC) of *Nepeta* FDE(10% and 15%) and *Nepeta* SDE (10% and 15%) were determined by the micro-well dilution assay according to the CLSI method (4), in tripticase soy broth pH 7.2 supplemented with 5 % fetal calf serum (FCS) and 0.01% (W/V) of 2,3,5-triphenyltetrazolium chloride as visual indicator of bacterial growth. The inoculum of each strain of *H. pylori* (NCTC 11638 and two clinical isolates) were prepared from 48 h of culture and the suspension was adjusted to the tube 0.5 of Mc Farland scale (10^8 bacterial cells). Then, they were diluted 10 times. The lyophilized of FDEs and SDEs were dissolved in distilled water to the highest concentration to be tested (10 mg/ml), and then serial two-fold dilutions were made in concentration ranges from 10 to 0.310 mg/ml. In addition, amoxicillin dilutions were prepared in a concentration range from 128 to 0.25 µg/ml. The 96-well plates were prepared by dispensing into each well 90 µl of nutrient broth and 10 µl of the inoculum. One hundred microlitre aliquot from the stock solutions of the aqueous extract and their serial dilutions initially prepared was transferred into five consecutive wells. The final volume in each well was 200 µl. The plates were covered with sterile plate sealer and then incubated at 37°C for 72 h under the microaerophilic condition. MIC was defined as the lowest concentration of the extracts in the medium in which there was no visible growth after incubation (no red colour). In addition controls of nutrient broth, strains and extracts were also included. The experiments were performed in duplicate and then replicated at least twice.

Determination of minimal bactericidal concentration (MBC)

Extracts that showed inhibitory activity in the preliminary broth assay were submitted to a subculture in Mueller-Hinton agar supplemented with 7% horse blood, in order to evaluate bacterial growth. The plates were incubated under the same conditions as in the MIC experiment. The presence or absence of bacterial growth was determined by visual inspection. The MBC was defined as the lowest concentration that showed no bacterial growth in the subcultures after incubation.

Results

Rheological analysis of dried products

The pharmaceutical technological properties of solid extracts were evaluated with the aim of obtaining appropriate solids for tablet preparation. The considered properties of the dried products were: product yielding, physical-chemical stability and compaction parameters.

The SDEs showing best pharmaceutical characteristics and the most suitable size and shape of particle was selected for the following assays. In Figure 1, SDE containing Aerosil™ in a concentration of 30 % (w/w) respect to the infusion solid residue is shown.

Chemical profile

SDE and FDE samples were analyzed by CE with the aim of obtaining a chemical profile of the components of the dried extracts. For all the cases the electrophoretic fingerprints were achieved in less than 20 minutes. The infusion, SDE and FDE profiles showed a similar chemical composition. However, at 6.1 min, a peak with analogous corrected area was detected in the infusion and FDE profiles; in addition it was not found in the SDE.

In the FDE samples the concentration of the main components was lower than in the infusion and SDE samples. The highest concentration of the metabolites was found in the SDE samples. The corresponding electropherograms are shown in Figure 2.

Anti-*H. pylori* activity

The reference strain NCTC 11638 is susceptible to CLA and MTZ (0.5 mg/ml and 0.25 mg/ml respectively); the clinical isolates assayed, were both HP796 and HP857, susceptible to MTZ (2 mg/ml and1 mg/ml) and only HP796 resistant to CLA (4 mg/ml). All strains were susceptible to AMX. The *N. cataria* FDEs 10% and 15% (w/v) were active and inhibited *H. pylori* with a MIC varying from 0.31 to 10 mg/ml (Table 1). The *N. cataria* FDE 15% inhibited all tested microorganisms at lower concentration tested (0.31 mg/ml). Only *Nepeta* FDE 10% showed the same values of MIC and MBC for the reference strain of *H. pylori* (Table 2). The MBC values for *Nepeta* FDE 15% were of 10 mg/ml (five fold higher than the corresponding MICs values) for all tested strains. In general, MIC values □'3f□'3fwere bacteriostatic. Higher concentrations of extract were needed to have bactericidal effect. On the other hand, of the *Nepeta* SDEs 10% and 15% (w/v), none of them inhibited *H. pylori* at any assayed concentration.

Discussion

Eradication of *H. pylori* is an important objective in overcoming gastric diseases. Many regimens are currently available but none of them could achieve 100% success in eradication and the antibiotic resistance of *H. pylori* is the most important reason for failure in its eradication (15).

Spray drying and freeze drying are suitable techniques which allow obtaining solids with several pharmaceutical advantages over the conventional liquid form. Pharmaceutical oral solid forms are more convenient in terms of dosage and stability, thus, the formulation of plant extracts as tablets is of interest. Spray drying of medicinal plants extracts is a convenient way to making dry powders, granules, or agglomerates from drug-excipient solutions and suspensions. However, the high drying temperature restricts its application in processing thermal sensitive materials. FDEs can present good quality, but the extremely high production cost has limited its wide application.

The *N. cataria* FDEs 10% and 15% (w/v) at 10 and 0.31 mg/ml were active against *H. pylori* strains evaluated, in accordance with several reports of other *Nepeta* species. The essential oils obtained from the aerial parts of *Nepeta camphorata* and *Nepeta argolica* exhibited significant antibacterial activity against *H. pylori* (11) and the nepetalactone compound of *Nepeta septemcrenata* showed significant antibacterial against grampositive and gramnegative bacteria (10). On the other hand, SDEs were not active against *H. pylori* at any concentration used in this assay.

From the CE chemical fingerprints achieved it can be noted that the major compounds in the SDEs and the infusion are similar with higher concentration for the SDE. In consequence, minor components of the infusion not present after the spray drying procedure can be related with the antibacterial activity. Increasing inlet temperature favored the physical properties as well as the product yielding, although volatile and thermolabile compounds can be eliminated.

After analyzing the chemical profile of the FDEs the presence of the major peak already observed in the infusion and SDEs was confirmed but in a lower concentration. Considering that the activity was only ascribed to the FDEs, components absent in

SDEs but present in the FDEs profiles were searched. These compounds could be responsible of the anti- *H. pylori* activity probably by producing synergistic effects with other components.

The chemical and physical properties of dried products depend on the techniques applied (9); therefore it is difficult to choose the most recommendable. An appropriate drying method should consider the cost of production, avoid undesirable changes and maintain the good quality of the final product. The freeze-drying process seems to keep the active antibacterial compounds but its application could be limited due to its high cost.

Our results showed that the *N. cataria* aqueous extracts have ability to inhibit growth of the *H. pylori.* Thus, *N. cataria* may be developed as a potential drug for *H. pylori* infection treatment. In this sense, further research aimed the identification and purification of anti-*H. pylori* components from the *N. cataria* are necessary as well as to determine the degree of toxicity of these extracts.

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Microorganisms	MIC (mg/ml)		MIC (mg/ml)	
	FDE 10%	FDE 15%	SDE 10%	SDE 15%
H. pylori NCTC 11638	10	0.31	NA	NA
H. pylori 796	NA	0.31	NA	NA
H. pylori 857	10	0.31	NA	NA

Table 1. Minimal inhibitory concentration (MIC) obtained for Nepeta FDE (10% and 15%) and Nepeta SDE (10% and 15%)

NA: not active at maximun concentration tested (10 mg/ml)

Microorganisms	MBC (mg/ml)	
	FDE 10%	FDE 15%
H. pylori NCTC 11638	10	10
H. pylori 796	NA	10
H. pylori 857	NA	10

Table 2. Minimal bactericidal concentration (MBC) obtained for Nepeta FDE (10% and 15%)

NA: not active at maximun concentration tested (10 mg/ml)

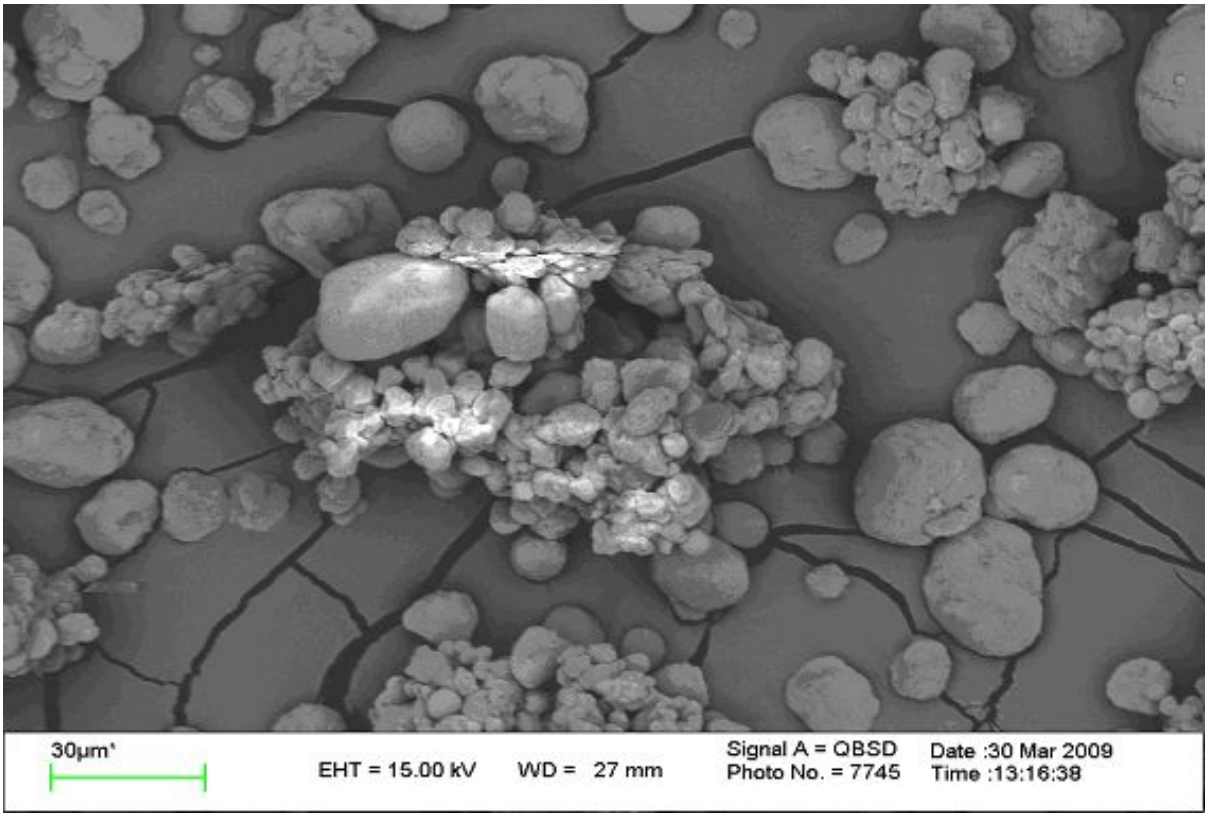

Figure 1: S.E.M. photomicrographs of SDE from infusion Nepeta cataria grounded leaves (10% w/v) in the presence of 30% (w/w) of colloidal silicon dioxide (Aerosil™)

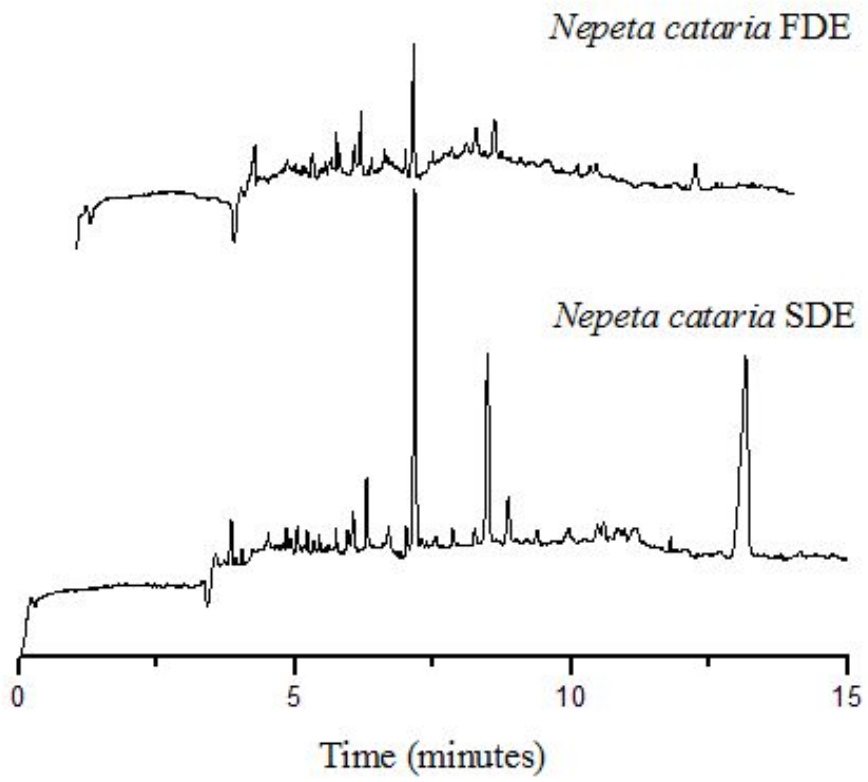

Figure 2: Electropherograms of FDE and SDE obtained from Nepeta cataria infusion (10% w/v) under the following conditions: BGE, tetraborate buffer 20 mM, pH 9.2, 25 kV applied voltage, 25 °C capillary temperature, 25 °C sample temperature; hydrodynamic mode sample injection, 0.5 Psi during 5 s; detection by DAD at 203 nm