



Validated HPLC Determination of the Potential Anti-*Helicobacter pylori*, Lepidine, in *Lepidium sativum* Seeds Assessed by Molecular Docking Study

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ABSTRACT

Background: Nearly 50 % of the world residents are known to be infected with *Helicobacter pylori* which is the main cause of peptic ulcer and gastric cancer. Since resistance to the currently used treatments, other alternative approaches such as combination with natural products should be tried. **Objectives:** In this work, validated HPLC-UV method was developed for quantification of lepidine content in various extracts and crude oil of *Lepidium sativum* seeds. Furthermore, antibacterial activity of lepidine against *H. pylori* was compared with standard antibiotics supported by molecular docking study. **Methods:** Different solvents were used to extract lepidine as well as the contents were determined using HPLC-UV method. Anti-*Helicobacter pylori* activity was examined using agar diffusion and dilution methods. Molecular docking study was done on *H. pylori* phosphoribosyltransferase enzyme. **Results:** The highest content of lepidine (316.91 $\mu\text{g mL}^{-1}$) was found in *n*-hexane extract. Lepidine exhibit antibacterial effect (MIC = 6.25 $\mu\text{g mL}^{-1}$) against *H. pylori* clinical isolates. The ability of lepidine to interact with amino acids in the phosphoribosyl transferase binding site might rationalize its observed activity. **Conclusion:** Our results demonstrate for the first time the anti-*Helicobacter pylori* activity of lepidine, which could therefore be developed as viable nutraceutical agent. Further investigations are required to formulate suitable pharmaceuticals to combat with the *H. pylori* infections on clinical grounds.

Keywords: *Helicobacter pylori*; HPLC-UV; Lepidine; *Lepidium sativum*; Molecular docking

INTRODUCTION

Helicobacter pylori is considered as the chief etiological cause of serious gastric diseases such as gastric ulcers and cancer¹. Several international guidelines for treating patients diagnosed with *H. pylori* infections were consistent with the use of triple therapy; a proton pump inhibitor, clarithromycin, and amoxicillin^{2, 3}. However, as clarithromycin resistance

is in increase, triple therapy produce lower than 80% cure rate⁴. Since antibiotics are expensive and induce side effects as well as there is no large-scale production of vaccine against *H. pylori*⁵, therefore, other natural alternative approaches should be tried. *Lepidium sativum* Linn. (Brassicaceae) is an edible fast-growing annual herb and economically important. *L. sativum* seeds have been extensively used in traditional medicine⁶. The bitter seeds are rubefacient,

thermogenic, galactagogue, emmenagogue, aphrodisiac and diuretic⁷. Seeds infusion is used to relieve cough, as carminative and in chronic liver enlargement⁶. Local application as poultices of crushed seeds with lime are used in rheumatic pains, useful for sprains, in leprosy and skin diseases⁷. A food supplement capsule containing *L. sativum* was tried on irregular bowel movements and constipation case, it was produce 90% symptoms improvement⁸. *L. sativum* seeds were reported to has chemoprotective⁹, antidiabetic¹⁰, antihypertensive¹¹, hepatoprotective, antihypercholesterolemic¹² and antioxidant activities⁶ as well as the clinical studies showed that it may be useful in treatment of bronchial asthma¹³.

Natural quinoline alkaloids and their derivatives possess significant activities; antitumor, antimalarial, antibacterial, insecticidal, antiviral, anti-inflammatory, herbicidal and other activities¹⁴. Quinine is the most famous quinoline alkaloid, and its discovery opened new areas in antimalarial drug development. Quinoline alkaloids were highly selective against *H. pylori* and has a unique antimicrobial mechanism (s)¹⁵. Lepidine (4-methylquinoline) is the main quinoline alkaloid in *L. sativum* seeds¹⁶.

The yields of extractable components are strongly influenced by polarity of the extraction solvent and the technique employed, in addition to their chemical nature¹⁷. Various trials to increase the micropropagation yield of *Lepidium* were done, in which the lepidine content was determined from *in vivo* and *in vitro* grown plants¹⁸. In this work, different solvents were used to extract lepidine as well as lepidine contents were compared in various extracts and crude oil of *L. sativum* seeds using validated HPLC-UV method. Furthermore, the antibacterial activity against *H. pylori* was evaluated and supported by docking study on *H. pylori* phosphoribosyltransferase enzyme.

MATERIAL AND METHODS

Plant material

L. sativum seeds were purchased from local herbalist. The identity was confirmed by Prof. Abd El-Halem A.E. (Agriculture Research Center, Cairo, Egypt) and a voucher specimen (LP# 1207) was kept at Faculty of Pharmacy, October 6 University.

Chemicals

Double distilled ultra-pure water prepared from Millipore water purification system (Bedford, USA). Methanol (HPLC grade) and lepidine standard (1g mL⁻¹) were purchased from Sigma-Aldrich Co., Germany. Sodium dihydrogen orthophosphate monohydrate, disodium hydrogen phosphate dihydrate and *n*-hexane (analytical grade) were purchased from El-Nasr Co., Egypt. Antibiotic discs (6-mm);

metronidazole (5 µg), clarithromycin (15 µg), tetracycline (10 µg), ciprofloxacin (5 µg) and amoxicillin (10 µg) were purchased from HiMedia Laboratories, Mumbai, India.

Helicobacter pylori isolates

Five isolates of *H. pylori* from biopsies of the gastric antrum (October 6 university hospital, Giza, Egypt) were identified by culturing directly onto Columbia blood agar with DENT supplement (Oxoid, UK) and incubating microaerobically (5% CO₂, 72 hr, 37 °C). *H. pylori* isolates were identified by colony morphology, observing the spiral shape on Gram staining, and positive urease, oxidase, and catalase tests.

Extraction processes and preparation of standard lepidine

n-hexane extract was prepared by sonication of powered seeds (1g) with *n*-hexane (20 ml, 30 min) in a closed volumetric flask, filtered and collected. The remaining cake was sonicated with methanol as in case of *n*-hexane. Crude oil was prepared by cold expression of *L. sativum* seeds (10 g) followed by filtration. Crude oil, *n*-hexane and methanol extracts were collected separately concentrated, filtered (millipore 0.2 µm), and injected into HPLC. Standard lepidine (working solution) was prepared by dissolving lepidine (1g mL⁻¹, 0.1 mL) in HPLC mobile phase (10 ml).

Chromatographic conditions

The chromatographic analysis was done using Shimadzu HPLC (LC-20AT series, Shimadzu, Japan) with multiple wavelength detector (SPD-20AD Model) controlled with LC solution version 1.2 (Shimadzu, Kyoto, Japan) for data acquisition and manipulation. Separation was achieved using ProntoSIL ODS C₁₈ (5 µm, 15 cm × 4.5 mm I.D.) column with 1 mL min⁻¹ flow rate, UV detection at 254 nm and the column oven was thermostated at 30 °C. Sodium dihydrogen orthophosphate monohydrate (0.325 g) and disodium hydrogen phosphate dihydrate (1.36 g) were dissolved into bidistilled water (1 L) to get phosphate buffer (pH 7.4). Isocratic elution was proceed using phosphate buffer: methanol (1:1 v/v) as a mobile phase.

Validation of HPLC method

Before application, The method was carefully validated according to guidelines provided by International Conference on Harmonization (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use^{19, 20}.

a) Linearity

Aliquots of lepidine working solution (1, 0.75, 0.5, 0.25 and 0.1 mL) were transferred into a series of volumetric flasks (10 mL) and complete the volumes

with the mobile phase. Samples, which their concentrations ranged from 100 to 1000 $\mu\text{g mL}^{-1}$, were analyzed using the same chromatographic conditions and calibration curve was constructed between the peak areas and the corresponding concentrations. Regression equation and the limit of quantification (LOQ) were estimated.

b) Accuracy (Recovery)

The accuracy of the results was checked by applying the proposed HPLC method for determination of different blind samples of standard lepidine. The concentrations were obtained from corresponding regression equation.

c) Precision

The degree of method repeatability was measured by analyzing three concentrations (250, 500 and 750 $\mu\text{g mL}^{-1}$) of lepidine three times intraday by the proposed HPLC method and the relative standard deviation (RSD) was calculated. Intermediate precision was measured by analyzing the same three concentrations three times interday by the proposed HPLC method and the RSD was calculated.

d) Robustness

Method robustness was assessed at two lepidine concentrations (500 and 750 $\mu\text{g mL}^{-1}$). The experimental conditions were deliberately altered, and the resolution of lepidine was recorded. The flow was changed by 0.2 units from 0.8 to 1.2 mL min^{-1} to study the effect of flow rate on the resolution. The effect of pH was studied by varying ± 0.1 pH units. Methanol percent was changed by ± 2 % to study the effect of organic strength on the resolution while other mobile phase component was held constant.

Calibration curve and method application

Calibration curve was designed with five concentrations of lepidine working solution after dilution (100, 250, 500, 750, 1000 $\mu\text{g mL}^{-1}$) with mobile phase and applied in triplicates into HPLC analysis (Figure 1). The HPLC developed method was applied for quantification of lepidine content in crude oil, *n*-hexane, and methanol extracts.

Evaluation of lepidine anti-*Helicobacter pylori* activity (Susceptibility test)

Antibacterial activity of lepidine was evaluated by the agar diffusion method using five *H. pylori* clinical isolates on Columbia blood agar^{21, 22}. An inoculum (10×10^7 CFU) of *H. pylori* isolates were used for the completed seeding. Aliquots (20 μL of each examined sample) were applied in wells (10 mm) made on the surface of media. After microaerophilic incubation (72 h, 37°C), the clear zones diameter were

measured. The susceptibility tests were performed in triplicates, and the results were presented as mean \pm SE of clinical isolates. Antibiotics disks; amoxicillin, tetracycline, clarithromycin, ciprofloxacin and metronidazole were placed on the bacterial plates, and inhibition zones were measured^{23, 24}. The clinical isolates of *H. pylori* were declared as sensitive or resistant by the zone of inhibition following the criteria of Clinical Laboratory Standards Institute^{21, 22}.

Determination of Minimum Inhibitory Concentration (MIC) of lepidine on *H. pylori*

The MIC was determined as the lowest concentration lepidine that completely inhibit the bacteria growth by agar dilution method in triplicates. Lepidine was dissolved in DMSO and serial dilution was proceed. Each bacterial suspension was spot inoculated (1×10^5 CFU/spot) onto Columbia agar base with 5% supplemented sterile sheep blood plates containing lepidine, and the MIC was determined following microaerophilic incubation (72 h, 37°C)^{21, 22}.

Molecular docking study

Docking study of lepidine was performed by Molecular Operating Environment (MOE 2014.0901) using Triangle Matcher placement method and London dG scoring function. The X-ray crystallographic structure of *H. pylori* phosphoribosyltransferase co-crystallized with the quinolinic acid (PDB ID: 2B7N)²⁵ was downloaded (<https://www.rcsb.org/>) and removed unnecessary chains A and C, water molecules and ligands. The enzyme and lepidine were 3D protonated and selecting the least energetic conformer. Docking setup was first validated by redocking quinolinic acid. The suitability of the running protocol was assured by low binding energy score (S -6.307 kcal/mol.), small RMSD (0.633 Å) and with the ability of the redocked pose to reproduce all the key interactions (Figure 3).

RESULTS

Validation parameters of HPLC method

HPLC is a well-known technique for separation and quantification of plant active components. Calibration curve was generated using standard lepidine solution. The method was carefully validated according to ICH guideline¹⁹ before application for lepidine quantification. Linearity was assessed by analysis of lepidine standard in triplicates and mean values used to construct the calibration curve with average correlation coefficient of 0.9997. The method shows good accuracy and repeatability with recoveries of 98.57 and 98.93 % respectively (Table 1).

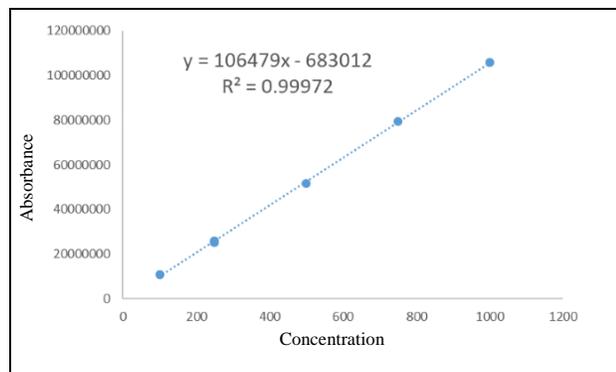


Figure 1. Calibration curve of standard lepidine

Table 1. Validation parameters for the HPLC lepidine determination method

Parameters	HPLC values
Accuracy (mean \pm S.D) ^a	98.57 \pm 1.12 %
Precision (RSD %):	
Repeatability ^b	98.93 %
Intermediate precision ^b	0.745
Linearity:	
Slope	106479
Intercept	- 683012
Correlation coefficient (R ²)	0.9997
Range ($\mu\text{g mL}^{-1}$)	100- 1000
Robustness (RSD %) ^c	1.311

^a Standard deviation (S.D) of the average of 3 determinations.

^b Intraday and interday (each of n=9) and average of three different concentrations.

^c RSD of the average concentration determinations after deliberate conditions change.

Lepidine identification and quantification by HPLC-UV method

It was commonly aware that the complexity of natural products made it extremely difficult to separate and determine its active constituents in crude drugs or in their medical preparations²⁶. The HPLC-UV chromatograms of *L. sativum* extracts showed one major peak, registered at retention time (11.43 min) identified as lepidine according to pre-examined standard solution (Figure 2). Other peaks in approximately the first few minutes of HPLC chromatograms probably correspond to interfering polar compounds. Lepidine was quantified from the calibration curve obtained from the standard solution. It can be observed that *n*-hexane can extract the high yield of lepidine (316.91 $\mu\text{g mL}^{-1}$). Crude oil and methanol extract contain 129.15 and 39.14 $\mu\text{g mL}^{-1}$, respectively.

Anti-*Helicobacter pylori* activity

The results cover up that, *n*-hexane extract was the most effective anti-*Helicobacter pylori* agent (50 \pm 0.7 mm zone of inhibition). Lepidine showed an inhibitory activity (31 \pm 1.5 mm) (Table 2). Activity of *n*-hexane extract was found to be higher than lepidine itself may owing to its content of other active phytoconstituents. Tetracycline showed an inhibitory activity (30 \pm 2.2 mm) while metronidazole showed less effect against *H. pylori* (14 \pm 2 mm). Sensitivity of *H. pylori* isolates were detected based on the guidelines provided by National Committee for Clinical Laboratory Studies (NCCLS)²⁷, which reported that the organisms being resistant to amoxicillin if its inhibition zone size \leq 18 mm; and resistant to ciprofloxacin, clarithromycin, and tetracycline if \leq 30 mm zone size while resistant for metronidazole if zone size \leq 16-mm²⁸. Resistances to ciprofloxacin, tetracycline and clarithromycin were found to be relatively high compared with lepidine (Table 2). It is well known that tetracycline is bacteriostatic against most susceptible bacterial pathogens²⁹ while metronidazole is often used to eradicate *H. pylori* along with other drugs³⁰. The MIC of lepidine was also tested by agar dilution method. None of the clinical isolates of *H. pylori* showed any growth around (300-6.25 $\mu\text{g mL}^{-1}$). As a result, the MIC of lepidine against *H. pylori* was 6.25 $\mu\text{g mL}^{-1}$, which is a good breakpoint concentration relatively with other antibiotics used in the same cases of *H. pylori* infections (Table 3).

Table 2. Effect of extract and standards against clinical *H. pylori* isolates by agar diffusion method

Extracts and Standards	Zone of inhibition * (mm)
<i>n</i> -hexane	50 \pm 0.7
Lepidine (100 mg mL ⁻¹)	31 \pm 1.5
Metronidazole (5 μg)	14 \pm 2.3
Clarithromycin (15 μg)	25 \pm 1.7
Tetracycline (10 μg)	30 \pm 2.2
Ciprofloxacin (5 μg)	20 \pm 1.7
Amoxicillin (10 μg)	23 \pm 2.4

* Tests were performed in triplicates and presented as mean \pm SE

Molecular docking of lepidine on *H. pylori* phosphoribosyltransferase enzyme

The validated setup was used in predicting the ligand-receptor interactions at the binding site for lepidine. The binding pocket is mainly composed of conserved basic residues (Arg125, Arg148, and His147), hydrophobic residues (Met156 and Met207)

and one threonine residue (Thr124). The main key interactions include hydrogen bonds between the anionic carboxylate groups of quinolinic acid and the side chains of Arg148 and Arg125 amino acids (Figure 3). Lepidine interacts with the side chain of Arg125 through H-bonding through its nitrogen atom. Also, extra-hydrophobic interactions of lepidine through its pyridine and benzene rings to Thr124 and Arg125 amino acids may stabilize the binding mode to the enzyme (Figure 3).

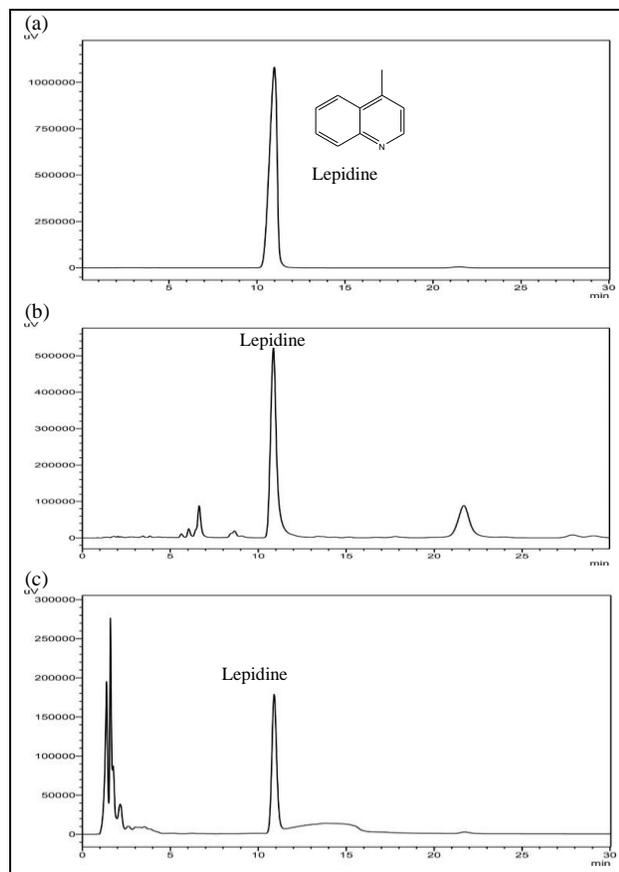


Figure 2. HPLC chromatograms of (a) *n*-hexane extract; (b) methanol extract; (c) crude oil of *L. sativum* seeds.

Table 3. The MIC of lepidine against clinical *H. pylori* isolates by agar dilution method

Lepidine concentration ($\mu\text{g ml}^{-1}$)	300	200	100	50	25	12.5	6.25	3.125
Growth	-	-	-	-	-	-	-	+

Discussion

Extraction and quantification of major phytochemicals in herbal medicines is a prerequisite in quality control. The extraction yield of metabolites fluctuates according to the solvent used. In this study, *n*-hexane and methanol were used to cover a broad range of solvent polarity. Generally, lepidine is sparingly soluble in water, but freely soluble in alcoholic and nonpolar solvents. Although methanol can successfully extract lepidine, large amounts of interfering compounds were also co-extracted. Consequently, these interfering compounds could adversely affect lepidine qualitative and quantitative analysis. Extraction with *n*-hexane decrease these interfering polar compounds.

According to WHO, *H. pylori* is documented as class 1 carcinogen. Treatment of *H. pylori* using synthetic compounds is difficult as high cost, bacterial resistance and adverse side effects. Therefore, exploration of safer natural *anti-Helicobacter pylori* phytochemical is becoming important. Sensitivity of *H. pylori* isolates was detected based on the guidelines provided NCCLS²⁷, resistances to ciprofloxacin, tetracycline and clarithromycin were found to be relatively high compared with lepidine. Lepidine shows a strong antibacterial effect at low MIC (0.006 mg mL^{-1}) in parallel with well-known marketing *H. pylori* antibiotics; ciprofloxacin and metronidazole (0.005 mg mL^{-1}). Quinolinic acid (QA) is the first NAD synthesis intermediate. Quinolinic acid phosphoribosyltransferase (*Hp*-QAPRTase), an essential enzyme in the NAD₂ biosynthesis, catalyzes phosphoribosyl moiety transfer from 5-phosphoribosyl-1-pyrophosphate to QA. The step, which is central to NAD biosynthesis and cell survival in prokaryotes, is thus represents an attractive target for antibacterial drugs²⁵. The ability of lepidine to interact with the key amino acids in binding site of *Hp*-QAPRTase enzyme might rationalize its observed activity as indicated by the docking pattern.

Quinoline alkaloids reduced the *in-vivo* bacterial growth by inhibited the bacterial respiration³¹. Quinoline alkaloids has a unique antimicrobial mechanism (s) different from those of other examined antibiotics. it may be beneficial in the treatment of *H. pylori*-associated gastroduodenal diseases^{15, 14}. This finding agreed with Hamasaki *et al.* who indicated that quinoline alkaloids were highly selective against *H. pylori*¹⁵. Our investigation may partially validate the use of *L. sativum* seeds but further bioactivity-guided investigations are required to formulate suitable pharmaceuticals to combat with the *H. pylori* infections on clinical grounds.

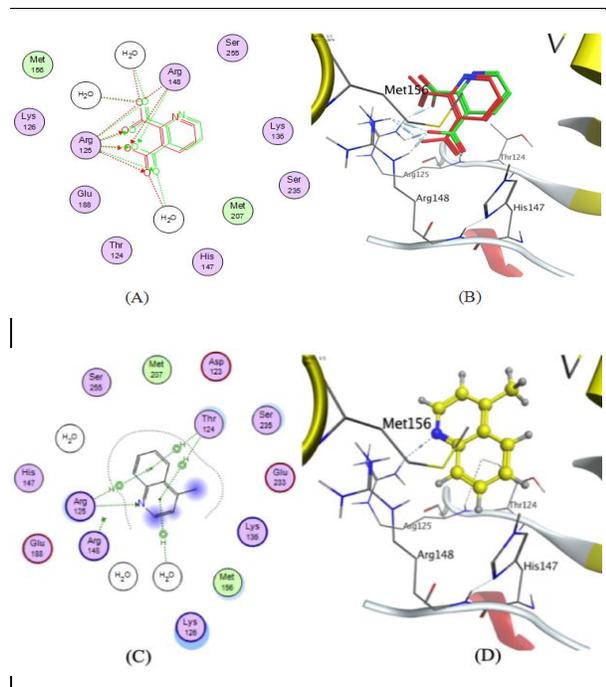


Figure 3. 2D diagram (A), 3D representation (B) showing superimposition of the co-crystallized ligand (red) and the re-docked pose (green), 2D diagram (C) and 3D representation (D) showing the binding mode of lepidine (yellow) with the enzyme binding pocket.

CONCLUSION

As increasing *H. pylori* resistance against synthetic drugs, the use of medicinal plants and/or their chemical components may have potential benefits. The obtained remarkable *anti-Helicobacter pylori* activity of lepidine may introduce *L. sativum* seeds as important nutraceutical that can be used in combination with other drugs to treat peptic ulcer. The simplicity of the HPLC method combined with excellent sensitivity, resolution, and short analysis time, makes it a useful tool for characterizing lepidine in nutraceuticals.

Conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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