Journal of Ethnopharmacology 183 (2016) 54–58

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Ethnopharmacological communication

Anti-*Helicobacter pylori* activity of bioactive components isolated from *Hericium erinaceus*

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ARTICLE INFO

Article history:
Received 23 December 2014
Received in revised form
1 September 2015
Accepted 5 September 2015
Available online 11 September 2015

Keywords:
Hericium erinaceus
Ethanol extracts
Low-molecular-weight bioactive components
Anti-*Helicobacter pylori* activity

ABSTRACT

Ethnopharmacological relevance: The fungus *Hericium erinaceus* (*Bull.*) Pers is used in Chinese traditional medicine to treat symptoms related to gastric ulcers. Different extracts from the fungus were assessed for anti-*Helicobacter pylori* activity to investigate the antibacterial activity of the ethanol extracts from *H. erinaceus* and verify the traditional indication of use.

Materials and methods: The fruiting bodies of *H. erinaceus* were concentrated with ethanol by HPD-100 macroporous resin and the whole extract was partitioned by petroleum ether and chloroform to afford fractions with using a silica gel column. Several pure compounds of petroleum ether extracts were obtained and analyzed using nuclear magnetic resonance (NMR). The activity of the extracts and fractions towards *H. pylori* was assessed by the microdilution assay and by the disk diffusion assay *in vitro*. From the most active fraction, two pure compounds were isolated and identified as the main components with anti-*H. pylori* activity from the fungus *H. erinaceus*. The cytotoxicity of these two compounds against the human erythroleu-kemia cell line K562 was also evaluated.

Results: The crude ethanol extracts from the fungus *H. erinaceus* were inhibitory to *H. pylori*. The petroleum ether extracts (PE1s, PE2s) and the chloroform extracts (TEs) demonstrated strong inhibition to *H. pylori*. The inhibition of *H. pylori* was observed through an agar dilution test with minimal inhibition concentration (MIC) values from 400 μg/mL to 12.5 μg/mL. Two pure compounds, 1-(5-chloro-2-hydroxyphenyl)-3-methyl-1-butanone and 2,5-bis(methoxycarbonyl)terephthalic acid were isolated from the petroleum ether fractions and identified using ^{1}H NMR and ^{13}C NMR spectra analysis. The MIC value for 1-(5-chloro-2-hydroxyphenyl)-3-methyl-1-butanone was 12.5–50 μg/mL and the MIC value for 2,5-bis (methoxycarbonyl)terephthalic acid was 6.25–25 μg/mL. Both two compounds showed weak cytotoxicity against K562 with $IC_{50} < 200$ mM.

Conclusions: This study revealed that the extracts from petroleum ether contribute to the anti-*H. pylori* activity. The compounds obtained from petroleum ether extracts, 1-(5-chloro-2-hydroxyphenyl)-3-methyl-1-butanone and 2,5-bis(methoxycarbonyl)terephthalic acid, inhibit the growth of *H. pylori*.

1. Introduction

Hericium erinaceus (*Bull.*) Pers (Houtoujun in China, yamabushitake in Japan and the lion's mane mushroom in the US) belongs to the Basidiomyeota, Basidiomycetes, Agariomycetes, Russulales, and *Hericiaceae* families and is the traditional medicinal food for the treatment of gastricism in China and Japan (Zhu et al., 2014; Li et al., 2012). Traditionally, *H. erinaceus* is a luxurious food in China, and a number of processed products derived from *H. erinaceus* flood the market. It contains important pharmacological constituents such as polysaccharides, bioactive proteins, terpenoids and hericenone. Previous studies have demonstrated that extractions from the fruiting bodies exhibit antitumor activity, hypoglycemic activity, anti-bacterial activity and anti-inflammatory properties (Sheu et al., 2013; Han et al., 2013; Patel and Goya, 2012). Li et al. (2014) demonstrated that the extracts from *H. erinaceus* were active against gastric cancer NCI-87 cells *in vitro* and tumor xenografts bearing in SCID mice *in vivo*, and these extracts had the potential for development into anticancer agents for the treatment of gastrointestinal cancer used alone and/or in combination with clinical used chemotherapeutic drugs. These studies have identified the bioactive polysaccharide. Other low-molecular-weight bioactive compounds have also exhibited

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http://dx.doi.org/10.1016/j.jep.2015.09.004
0378-8741/

Table 1
Growth inhibition of *H. pylori* by *H. erinaceus* ethanol extracts.

Tested sample[a]	*H. pylori* ATCC 43504	*H. pylori* SS1	*H. pylori* DXF	*H. pylori* W_2504	*H. pylori* 9	*H. pylori* 64	*H. pylori* 78	*H. pylori* 83
CK	−	−	−	−	−	−	−	−
EEs	+	+	+	+	+	+	+	+
PE1s	++*	+	+	+	+	++*	+	+
PE2s	+	+	+	+	+	+	+	+
TEs	+	+	+	+	+	+	+	+
MTZ	+++	+++	+++	+++	+++	+++	+++	+++

(−) not effective; (+) slightly effective; (++) moderately effective; and (+++) highly effective.

* Statistically singnificant at $P < 0.05$. $n=7$ in each group. The inhibition zone values of the extracts were compared to determine variation in their efficacy against the isolates.

[a] 75% EtOH in volume (CK); ethanol extracts (EEs); first petroleum ether extracts (PE1s); second petroleum ether extracts (PE2s); chloroform extracts (TEs); MTZ, metronidazole.

bioactivity. Hericenone L, isolated from *H. erinaceus*, demonstrated anti-cytotoxic activity on the EC109 cell line (Ma et al., 2012a). Amycenone, in *H. erinaceus,* increased intracerebral NGF and reduced depression and anxiety (Inanaga, 2012). Ergosterol peroxide, isolated from *H. erinaceus*, inhibited the growth of *Staphylococcus atzreus, Bacillus megaterium, Bacillus thuringiensis, Bacillus subtilis* and *Escherichia coli* (Ma et al., 2012b).

Helicobacter pylori is an important factor in gastric disease. The identification of an anti-*H. pylori* substance could be used to treat gastric disease (Wu et al., 2014; Chang et al., 2012). There are many therapeutic strategies, including chemotherapy that can treat gastric disease. However, these treatments are systemically toxic and drug resistance has limited their success. New therapeutic strategies, with improved immunity potential less toxic side effects, are being developed to treat gastric disease. Wang et al. (2008) reported that the *H. pylori* exterminate rate with the Houtoujun tablet was significantly higher than that with sesapride (216 cases of clinical validation). A low dose of decolored polysaccharides significantly improved the repair of a gastric mucosal injury (Jiang et al., 2014). The flavonoids isolated from *Piper carpunya* contributed to the anti-*H. pylori* activity. The Bi^{3+} with lower content to HEP from *H. erinaceus* exhibited strong inhibition effects on *H. pylori* (Quílez et al., 2010; Zhu et al., 2014). The methyl antcinate B, antcins A and K, from the fruiting bodies of *A. camphorata*, demonstrated anti-*H. pylori* activity (Geethangili et al., 2010). Haiying Rong et al. (2012) reported that the *H. pylori* exterminate rate of *H. erinaceus* with antibiotics was superior to antibiotics alone in the treatment of patients with peptic ulcer, with reduced recurrence.

Previously, we examined the antimicrobial activity of the ethanol extracts of 14 species of mushrooms using *H. pylori*. The extract of *H. erinaceus* inhibited *H. pylori in vitro* as demonstrated by the minimal inhibition concentration test (MIC < 10 mg/mL) *in vitro* (Shang et al., 2013). This study evaluated the antibacterial effects of the compounds on anti-*H. pylori* and antineoplastic activity against human erythroleukemia cell lines K562.

2. Materials and methods

2.1. Materials and reagents

The fruiting bodies of *H. erinaceus* were obtained at Fengxian District, Shanghai, China, and authenticated by Dr. Shang Xiaodong at the Institute of Edible Fungi, Shanghai Academy of Agricultural Science, China. A voucher specimen (*H. erinaceus* 0605) was deposited at the Institute of Edible Fungi, Shanghai Academy of Agricultural Sciences (Dr. Tan Qi, 2012, Shanghai, China). Analytically pure dry powders of MTZ (metronidazole) and TET (tetracycline) were purchased from Amresco (Solon, OH, USA). Chemical grade reagents were purchased from China National Pharmaceutical Group Corporation (Sinopharm).

2.2. Preparation of extracts

The entire fruiting body (200 g) was macerated with 2 L deionized water at room temperature. Chemical grade ethyl alcohol (95%) was used for extraction. Macerated material (100 mL) was mixed tenfold in HPD-100 macroporous resin (Cangzhou Bon Adsorber Technology Co., Ltd. Cangzhou, Hebei Province, China). The mixtures were absorbed and concentrated on a constant temperature shaker (Shanghai Sukun Industry & Commerce Co., Ltd. Shanghai, China) at 110 rpm and 25 °C for 24–48 h. They were eluted with three times the column bed volume (BV) of water. Ethanol (75%, 50 mL) was added to the concentrate and filtered at 110 rpm and 25 °C on the constant temperature shaker for 2 h. The ethanol was evaporated to obtain the crude extract. Bioassay-guided results indicated that the ethanol fraction was efficient at inhibiting *H. pylori* (Table 1).

The crude ethanol extract (100 mL) was at reflux (5 mL of 1 g mL^{-1} KOH and a small amount of zeolite at 80 °C) for 1 h using a condensation recycling installment to remove the saponification solvent. The slurry was cooled to room temperature (RT) and then successively extracted by petroleum ether to yield PE1s. The other solution was titrated to a pH between 2.0 and 3.0 using concentrated sulfuric acid. The liquid was extracted by petroleum ether to obtain PE2s and the extraction residue was concentrated with chloroform to yield the TEs. The anti-*H. pylori* activity of PE1s, PE2s, and TEs was determined.

2.3. Isolation of the active compounds

The active PE2s extraction (4 g) was analyzed on a CC (silica gel, 50 g; column, 100–200 mesh), then eluted with a petroleum ether–C_3H_6O gradient (50:1–0:1, v/v) using a thin-layer chromatography (TLC) control. It was then eluted with methyl alcohol. Ten fractions were obtained (II-1-6, 215.7 mg; II-7-9, 319.9 mg; II-10-13, 306.1 mg; II-14-18, 311.0 mg; II-19-30, 355.1 mg; II-31-45, 363.9 mg; II-46-53, 184.5 mg; II-54-58, 96 mg; II-59-63, 78.1 mg and II-64-78, 425.4 mg). The anti-bacterial activity of these components was determined (Table 2). The subfractions were evaporated by rotary evaporators and volatilized under fume cupboards at room temperature. Fractions II-10-13 and II-54-58 were recrystallized with acetone after Sephadex LH-20 to yield Compound **1** and Compound **2**.

Table 2
Activity of *H. erinaceus* extracts and most active compounds against *H. pylori* isolates. Results are presented as MIC values (μg/mL).

	H. pylori ATCC 43504	*H. pylori* SS1	*H. pylori 9*	*H. pylori* 64	*H. pylori* 78	*H. pylori* 83	*H. pylori* $W_2$504	*H. pylori* DXF
PE2s	250	500	250	500	500	500	250	500
II-1-6	100	50	50	100	100	50	200	100
II-7-9	200	100	100	100	100	25	200	50
II-10-13	100	25	25	100	100	25	50	100
II-14-18	50	50	12.5	50	25	25	50	50
II-19-30	12.5	25	25	12.5	25	12.5	25	25
II-31-45	25	50	25	50	25	25	25	25
II-46-53	100	25	50	100	100	50	200	50
II-54-58	> 400	200	100	> 400	200	100	200	100
II-59-63	50	50	100	50	100	50	100	100
II-64-78	100	100	200	100	200	100	> 400	200
Compound **1**	25	–	25	50	25	12.5	50	50
Compound **2**	12.5	–	6.25	12.5	25	12.5	12.5	6.25
MTZ	0.78	–	1.5625	1.5625	0.78	0.78	1.5625	1.5625
TET	3.125	–	3.125	1.5625	0.78	0.78	1.5625	1.5625

MTZ, metronidazole; TET, tetracycline.

(1) Compound **1**
Zhang et al. (2015) have isolated this compound and described the spectroscopic and spectrometric characteristics.
(2) Compound **2**

2,5-bis(methoxycarbonyl)terephthalic acid, a white transparent crystal, molecular formula, $C_{12}H_{10}O_8$,^{1}H NMR (500 MHz, CD_3OD): δ 7.34(1 H, s, CH),3.92 (3 H, s, OCH_3);^{13}C NMR (500 MHz, CD_3OD): 57.2 (C-9, 12), 107.1 (C-3, 6), 117.1 (C-2, 5), 131.5 (C-1, 4), 157.8 (C-5, 8), 169.3 (C-7, 10).

2.4. Bacterial strains and culture conditions

Eight strains, provided by Prof Guo, X.K., contained a standard strain *H. pylori* ATCC 43504 and a clinical standard strain *H. pylori* SS1. Six clinical isolates of *H. pylori* DXF: *H. pylori* $W_2$504, *H. pylori* 9, *H. pylori* 64, *H. pylori* 78 and *H. pylori* 83 were obtained from Shanghai Jiao Tong University, and the stock cultures were stored at −80 °C in milk. The strains were routinely inoculated into Brucella blood agar plates and supplemented with 8% sheep serum in a tri-gas incubator (5% CO_2 and 10% O_2) at 37 °C (Mégraud and Lehours, 2007).

2.5. Anti-Helicobacter pylori activity

The minimal inhibition concentration (MIC) was determined using the agar dilution test (Ndip et al., 2007). Different compounds were dissolved in 75% alcohol (v/v) to obtain a series of stock solutions at given concentrations. These solutions were added to 100 μL of Brucella blood agar broth with 8% sheep serum in a double grade dilution per well. *H. pylori* was inoculated at the beginning of the exponential growth phase (1×10^8 CFU/mL) after 1 day. The plates were incubated in a tri-gas incubator (5% CO_2 and 10% O_2) at 37 °C and the inhibition of bacterial growth was observed after 72 h of culture. The negative control was 75% alcohol, and metronidazole (MTZ) and tetracycline (TET) were used as the positive controls.

2.6. Cytotoxicity

Human erythroleukemia cell line K562 was purchased from American Type of Culture Collection (ATCC). Cells were grown in DMEM, supplemented with 10% PBS, 100 mg/mL of streptomycin, 100 units/mL of penicillin. Cells were incubated under 5% CO_2 humidified atmosphere at 37 °C.

The effect of Compound **1** and Compound **2** from *H. erinaceus* on cell viability was assessed by the MTT assay. The K562 cells were plated in 96-well plates at concentration of 105 cells/well and incubated for 24 h, then treated with various concentrations of Compound **1** and Compound **2**, which diluted with culture medium to give the appropriate final concentrations of 12.5–200 mg/mL. 5-Fluoro-2,4-(1h, 3h)-pyrimidinedione (5-FU) used as positive control which were dissolved in dimethyl sulfoxide (DMSO). Controls were done with the same final DMSO concentration in the medium as samples. After incubation of the cells with compounds 1–6 for 48 h at 37 °C, 20 mL of MTT (5 mg/mL) solution were added to each well and incubated for 4 h at 37 °C. Then the broth was removed and DMSO (150 mL) was added to each well. The degree of formazan formation, an indicator of living and metabolically active cells, was determined using ELISA reader at 570 nm. Time point was measured in three different experiments.

2.7. Statistical analysis

Each experiment was repeated more than three times and the results were identical. All data were expressed as the mean ± SE. The ANOVA test was used to calculate the statistical significance of the experimental results and to determine paired data compared with untreated controls. The symbol * indicates $P < 0.05$. *P*-values were used with the software IBM SPSS statistics 19.0 for Windows.

3. Results and discussion

3.1. Growth inhibition of Helicobacter pylori by H. erinaceus extracts

Previously, the anti-bacterial activity of ethanol extractions was assessed for 14 edible fungi: *H. erinaceus, Ganoderma lucidum, Cordyceps militaris, Pleurotus eryngii, Pleurotus ostreatus, Agrocybe aegerita, Lentinus edodes, Agaricus brasiliensis, Agaricus bisporus, Coprinus comatus, Grifola frondosa, Phellinus igniarius, Flammulina velutipes, and Hypsizygus marmoreus*. We determined that *H. erinaceus* significantly affected the inhibition to growth of *H. pylori*. This study assesses the anti-*H. pylori* activity of the extracts and fractions obtained through bioassay-guide fractionation and the isolation and structure elucidation of the active constituents from *H. erinaceus.*

The antibiotic activity of ethanol extracts was lower than that of the petroleum ether extracts (PE1s, PE2s) and the chloroform

extracts (TEs). We also evaluated the metronidazole (MTZ) inhibitory activity against *H. pylori* growth using the agar disk diffusion approach.

The petroleum ether and the chloroform extracts originating from the ethanol extracts of *H. erinaceus* inhibited the growth of some *H. pylori* strains. This result suggested that *H. erinaceus* may be a promising source of anti-*H. pylori* substances. Our data indicates that the extraction by PE1s, PE2s and the TEs resulted in greater activity than the ethanol extractions. However, there was a different gap contrast in the MTZ inhibitory activity against *H. pylori* growth. The concentration of the ethanol extracts (EEs), petroleum ether extracts (PE1s, PE2s) and the chloroform extracts (TEs) from *H. erinaceus* was 5 mg/ml. The standard antimicrobial agents, including MTZ (5 mg/ml), were used as positive controls. Ethanol (75%) was used as a negative control.

3.2. Bioactivity data of fractions II (minimum bactericidal concentration values)

We then evaluated the inhibitory activity against *H. pylori* growth for the fractions from PE2s using the agar disk diffusion approach. To determine whether *H. erinaceus* extracts inhibited *H. pylori*-growth, we analyzed PE2s extracts on a silica gel column, eluting with a 10 gradient and Sephadex LH-20 with methanol to yield Compounds **1** (31.6 g) and **2** (22.6 mg). The MIC values for the 10 fractions against seven clinical isolates (*H. pylori* SS1, *H. pylori* DXF, *H. pylori* W_2504, *H. pylori* 9, *H. pylori* 64, *H. pylori* 78 and *H. pylori* 83) and one reference *H. pylori* strain ATCC 43504 are given in Table 2.

The MIC values ranged from 250 μg/mL to 500 μg/mL for PE2s (Table 2). However, after silica gel column chromatography separation and thin layer chromatography analysis, the separated components from PE2s have obvious differences. The 10 components were II-1-6(215.7 mg), II-7-9(319.9 mg), II-10-13(306.1 mg), II-14-18(311.0 mg), II-19-30(355.1 mg), II-31-45(363.9 mg), II-46-53(184.5 mg), II-54-58(96.0 mg), II-59-63(78.1 mg) and II-64-78 (425.4 mg). The MIC for II-1-6 to the eight *H. pylori* strains ranged from 25 μg/mL to 200 μg/mL; 25–100 μg/mL for II-10-13; 12.5–50 μg/mL for II-14-18 and 12.5–25 μg/mL for II-19-30. For II-31-45, the MIC was 25–50 μg/mL; 25–200 μg/mL for II-46-53; more than 100 μg/mL for both II-54-58 and II-64-78 and 50–100 μg/mL for II-59-63. The inhibitory effects on identical strains differed for the different components. The MICs of some components were different and the inhibitory effects on different *H. pylori* strains illustrated that some components had specific inhibitory effects on *H. pylori*. To determine their specific composition and efficient active substances in the material, we continued further separation.

3.3. Compounds 1 and 2

In our study the lowest MIC value was 12.5 mg/mL for II-14-18 and II-19-30. Fractions II-14-18 and II-19-30 were the most potent extracts. The results suggest that Compound **1** and Compound **2** are potential sources of new antibacterial agents that are effective against some resistant strains. Compounds **1** and **2** were identified as 1-(5-chloro-2-hydroxyphenyl)-3-methyl-1-butanone), and 2,5-bis(methoxycarbonyl)terephthalic acid, respectively using ^{1}H NMR and ^{13}C NMR. Although various compounds were obtained from *H. erinaceus*, our study investigated Compounds **1** and **2** from antibacterial fractions. The *in vitro* growth inhibition assay *in vitro* against *H. pylori* was performed according to the agar dilution method, and the results indicated that the MICof Compounds **1** and **2** against one reference (ATCC 43504) and six clinically isolated *H. pylori* strains were 12.5–50 μg/mL and 6.25–25 μg/mL (Table 2). The MIC of the positive references metronidazole (MTZ) and tetracycline (TET) against these strains was 0.78–1.5625 μg/mL and 0.78–3.125 μg/mL, respectively (Table 2). The net content of Compound **1** is 31.0 mg and the net content of Compound **2** is 22.6 mg, from 200 mg (fruit body) of *H. erinaceus*.

Our research indicated that the o-hydroxyphenyl segments of the compounds demonstrated specific affinity with the FabI gene of the ENR enzyme, coenzyme NAD^+. This pathway resulted in pathogen elimination (Perozzo et al., 2002; Ward et al., 1999; Sivaraman et al., 2003). Zhu et al. (2010) demonstrated that the title compounds had a 92% inhibitory rate against *Monilia albican* and *Escherichia coli* at a mass concentration of 0.01% and antibacterial activity against *Staphylococcus aureus* (over 82%). Zhang et al. (2009) demonstrated that the o-hydroxyphenyl segments had a 100% inhibitory rate against *Monilia albican* at a mass concentration of 0.05% and antibacterial activity against *E. coli* and *S. aureus*.

A compound's antibacterial activity was enhanced by halogen groups such as Cl and Br. The activity was reduced by electron-donating groups of the phenylethanone rings, such as CH_3. The halogen atoms may enhance the hydrophobicity, and the electron clouds become delocalized. The preliminary bioassay for the two most potent plant extracts reported in our study was in the ranges of these studies. However, the inhibition of *H. pylori* has not been reported.

The average MIC values (12.5–50 μg/mL) (Table 2) and the anti-*H. pylori* activity of *H. pylori* 83 for Compound **1** are promising. Compound **2** demonstrated interesting anti-*H. pylori* properties, although II-54-58 resulted in low bioactivity. The inhibitory effects on *H. pylori* were the result of the o-hydroxyphenyl segment.

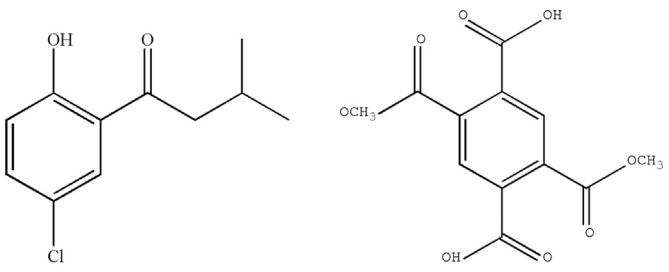
Compounds **1** and **2** were evaluated for their cytotoxicity against the human erythroleu-kemia cell line K562 by the MTT method reported (Alley et al., 1988). The cytotoxicity of compounds **1** and **2**, preformed as IC_{50} in mM. IC_{50} of Compound **1** was 733.2 ± 2.5 mM and that of Compound **2** was 422.8 ± 1.8 mM, while for the positive control, IC_{50} of 5-FU was 62.5 ± 1.4 mM. In conclusion, both compound **1** and compound **2** showed weak cytotoxicity against K562 with $IC_{50} > 400$ mM (Fig. 1).

Compounds **1** and **2** had similar levels of activity against the isolates of *H. pylori*. These results suggest that some fractions of the crude extracts possess antimicrobial activity.

To the best of our knowledge, this study is the first report of the anti-*H. pylori* activity of the ethanol extracts from *H. erinaceus*. The *H. erinaceus* extract demonstrated suitable inhibition to *H. pylori*, and may be a novel pharmaceutical approach against gastrointestinal disease.

Acknowledgments

The work was co-financed by Grants from the National Basic Research Program of China (973 Program) (No. 2012CB721102), the National Basic Research Program of China, the National Natural Science Foundation of China (No. 30830002), the Research Unit Fund of the Li Ka Shing Institute of Health Sciences (No. 7103506) for initiation of research.



Compound **1** Compound **2**

Fig. 1. Chemical structures of Compounds **1** and **2**.

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