NPC Natural Product Communications

2015
Vol. 10
No. 0
1 - 3

Anti-*Helicobacter pylori* Activity of Four *Alchemilla* Species (Rosaceae)

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Received: March 19th, 2015; Accepted: May 29th, 2015

The present study evaluated the anti-*Helicobacter pylori* activity of *Alchemilla glabra* Neygenf. (*A.* sect. *Alchemilla*), *A. monticola* Opiz (*A.* sect. *Plicatae* S.E. Fröhner), *A. fissa* Günther & Schummel (*A.* sect. *Calycinae* (Buser) Buser) and *A. viridiflora* Rothm. (*A.* sect. *Calycinae*), and identified ellagic acid and quercetin-3-*O*-β-D-glucoside. Anti-*H. pylori* activity was tested against ten clinical isolates and one reference strain (ATCC 43504). The methanol extracts were more active than the dichloromethane and cyclohexane extracts. The ranges of concentrations were between 4 μg/mL for methanol extracts of *A. viridiflora*, *A. glabra* and *A. monticola*, and 256 μg/mL for cyclohexane extracts of *A. viridiflora*, *A. glabra* and *A. fissa*. The best overall activity was obtained with *A. monticola* extracts. No significant difference was found in the ellagic acid contents of the methanol extracts of the tested *Alchemilla* species (0.2-0.3 mg/mL), and anti-*H. pylori* activity was similar (4 μg/mL-32 μg/mL). Ellagic acid exhibited strong activity at very low concentrations (0.125-0.5 μg/mL), while the second identified compound, quercetin-3-*O*-β-D-glucoside, was also very active in concentration of 2-16 μg/mL.

Keywords: *Alchemilla* sp., Ellagic acid, *Helicobacter pylori*, Quercetin-3-*O*-β-D-glucoside.

Helicobacter pylori (*H. pylori*), a ubiquitous human pathogen, is a Gram-negative bacterium associated with the development of chronic gastritis, peptic ulcer disease, and gastric cancer [1,2]. Standard antibiotic therapy is often not effective due to the high resistance of *H. pylori* [3]. Plants are important sources of biologically active compounds with significant antimicrobial properties. In particular, tannin containing plants have been used in the treatment of gastric diseases not only because of their astringent effect on gastric mucosa, but also because of anti-*H. pylori* activity. Water soluble tannins, catechins and proanthocyanidins, as well as ellagitannins, have been shown previously to have anti-*H. pylori* activity at low concentration [4-7]. Ellagic acid is a potent competitive inhibitor of gastric H^+, K^+-ATPase, and thus markedly inhibits acid secretion, and stress-induced gastric lesions. Enzyme inhibition was also evident for tannic acid [8,9].

Various species of *Alchemilla* L. (Rosaceae) are used in traditional medicine to treat the symptoms of sore throat, for wound healing, against bleeding, nausea and vomiting [10]. *Alchemilla* species have potent free radical scavenging activity, attributed to the phenolic compounds, tannins, and flavonoid glycosides [11-13]. *A. vulgaris* s.l. has been reported to possess ellagitannins (agrimoniin, laevigatin F, pedunculagin), flavonoids, essential oil, phytosterols and aliphatic hydrocarbons [13-15].

While the antioxidant, antimicrobial and neutrophil-modulating activities of *A. glabra* have been studied, anti-*Helicobacter* and other biological activities have not been described previously [16]. The present study was carried out to evaluate the anti-*H. pylori* action of *A. glabra* Neygenf. (*A.* sect. *Alchemilla*), *A. monticola* Opiz (*A.* sect. *Plicatae* S.E. Fröhner), *A. fissa* Günther & Schummel (*A.* sect. *Calycinae* (Buser) Buser) and *A. viridiflora* Rothm. (*A.* sect. *Calycinae*), and identified compounds.

The total tannin content in the methanol extracts of the tested *Alchemilla* species was 4.8-12.3%, calculated as pyrogallol, while the quantity of ellagic acid was between 0.2-0.3 mg/mL, which was lower than the results of other authors for *A. vulgaris* s.l. extract (Table 1) [17,18]. Besides ellagic acid, quercetin-3-*O*-β-D-glucoside was identified in the methanol extracts of *A. monticola* and *A. viridiflora.*

Table 1: The content of tannins (%) and ellagic acid (mg/mL) in methanol extracts.

Plant species	Tannins* (%)	Ellagic acid (mg/mL)
A. glabra	8.2	0.3
A. fissa	12.3	0.2
A. viridiflora	4.8	0.2
A. monticola	8.5	0.2

* The results were expressed as the percentage of pyrogallol.

Anti-*H. pylori* activity was tested against ten clinical isolates and one reference strain (ATCC 43504). The activity of the extracts is presented in Table 2. The methanol extracts were more active than the dichloromethane and cyclohexane extracts. The range of concentrations was from 4 μg/mL for methanolic extracts of *A. viridiflora*, *A. glabra* and *A. monticola* to 256 μg/mL for

Table 2: Anti-*Helicobacter pylori* activity of methanol, dichlormethane and cyclohexane extract of selected *Alchemilla* species (MIC, µg/mL).

Strains	*A. viridiflora*			*A. fissa*			*A. glabra*			*A. monticola*			M*	C**
	MeOH	CH_2Cl_2	C_6H_6	MeOH	CH_2Cl_2	C_6H_6	MeOH	CH_2Cl_2	C_6H_6	MeOH	CH_2Cl_2	C_6H_6		
1.	4	128	128	8	64	128	4	64	64	4	16	8	S	R
ATCC 43504	16	64	128	16	128	128	16	128	128	8	64	64	R	S
2.	8	64	128	32	128	256	32	128	128	4	64	32	S	S
3.	4	64	64	4	64	64	4	64	64	4	32	32	S	S
4.	4	64	128	4	64	128	4	64	256	4	32	64	S	R
5.	4	128	128	8	128	256	4	128	256	8	64	64	R	S
6.	4	64	128	16	128	256	16	128	128	8	64	64	S	S
7.	4	16	128	4	64	256	4	64	128	16	32	32	S	R
8.	4	64	64	4	64	64	4	64	64	8	32	32	R	R
9.	8	128	128	8	256	256	32	256	128	8	64	64	R	R
10.	8	128	256	16	256	256	8	256	256	16	64	64	S	S

*MIC-minimal inhibitory concentration, **Metronidazole, ***Claritromycin; 1-10 – clinical isolates of *Helicobacter pylori*; MeOH – methanol, CH_2Cl_2 –dichloromethane, C_6H_6 – cyclohexane.

Table 3: Anti-*Helicobacter pylori* activity of ellagic acid and quercetin-3-O-β-D-glucoside (MIC µg/mL).

Strains	Ellagic acid	Quercetin-3-*O*-β-D-glucoside	M*	C**
ATCC 43504	0.5	8	R	S
1	0.5	16	S	R
2	0.5	16	S	S
3	0.25	4	S	S
4	0.125	4	S	R
5	0.25	16	R	S
6	0.5	16	S	S
7	0.125	4	S	R
8	0.25	4	R	R
9	0.125	8	R	R
10	0.25	2	S	S

1-10 – clinical isolates of *Helicobacter pylori*; *Metronidazole, **Claritromycin.

cyclohexane extracts of *A. viridiflora*, *A. glabra* and *A. fissa*. The best overall activity was obtained with *A. monticola* extracts.

Ellagic acid exhibited strong activity in very low concentrations (0.125-0.5 µg/mL), while the second identified compound, quercetin-3-*O*-β-D-glucoside was also very active in concentrations of 2-16 µg/mL (Table 3). Previously, ellagic acid has shown activity against *H. pylori* ATCC 10231 with MIC values of 0.125 mg/mL, which was in accordance with our results, and MBC values against *H. pylori* G21 and 10K of 2 µg/mL and 10 µg/mL, respectively [5,19]. In the same experiment, quercetin-3-*O*-β-D-glucoside possessed very low activity against *H. pylori* G21 and 10K with MBC 480 µg/mL and 240 µg/mL, respectively [19].

The high activity of the methanol extracts could be explained by the presence of tannins, especially ellagic acid and ellagitannins. Also, the high activity of quercetin-glucoside could not be disregarded.

Experimental

Plant extract preparation: Aerial parts of *A. fissa* and *A. viridiflora* were collected in July 2013 at Mt Suva Planina (Serbia), while *A. monticola* and *A. glabra* were collected in August 2013 from Mt Stara Planina (Serbia). Voucher specimens (20130708/1-2, 20130708/1-2) were deposited in the Natural History Museum (Belgrade, Serbia) and identification of plant material was made by Dr Marjan Niketić. The air-dried, powdered material (380 g of *A. fissa*; 320 g of *A. viridiflora*; 53 g of *A. glabra*; 8.27 g *A. monticola*) were extracted with cyclohexane, dichlormethane and methanol, successively. The solvents were evaporated under low pressure to yield extracts.

Quantification of tannins: The official spectrometric method from Ph. Eur. 7.0 was used for quantification of tannins in the methanol extracts [20].

Quantification of ellagic acid: Ellagic acid content was determined in methanol extracts (2 mg/mL). HPLC separation was performed using an Agilent 1200 Series system equipped with a G1311A quaternary pump, a G1329A injector and G1315D DAD detector. The following conditions were used: column Zorbax Eclipse XDB-C18 (4.6 x 250 mm, 5 µm); column temperature 25°C; injection volume 5 µL; a flow rate 0.350 mL/min; detection at 256 and 367 nm. The mobile phase (A) water + 0.1% formic acid and (B) acetonitrile was used with a linear gradient: 0-2 min from 10 to 20% (B), 2-4.5 min from 20 to 90% (B), 4.5-4.8 min 90% (B), 4.8-4.9 min from 90 to 10% (B), 4.9-12.0 min 10% (B). The calibration curve was constructed from standard solutions of ellagic acid in methanol in the concentration range 0.017-0.55 mg/mL. The relationship between the peak area and concentration of ellagic acid was linear with a regression equation y=73579.63x+1232.5472. The correlation coefficient value corresponding to the calibration curve was r^2=0.998. Each measurement was performed in triplicate. The same chromatographic conditions were used for qualitative analyses of quercetin-3-*O*-β-D-glucoside.

Anti*-Helicobacter pylori *activity: Ten clinical isolates of *H. pylori*, including one highly resistant to metronidazole (M) (MIC > 16 ng/ml), 3 resistant to claritromycin (C) (MIC > 1 ng/ml), 2 resistant to both M and C, and 4 C and M susceptible, were tested. A reference strain of *H. pylori* (ATCC 43504) was used as a control. The strains were maintained at -80° C in Wilkins Chalgren Broth (Difco) with 10%, v/v, horse serum (Seromed) and 20%, v/v, glycerol (Merck) until required for experiments. Before being used, the strains were sub-cultured twice on Columbia agar base (Difco) supplemented with 10% horse serum and 0.25% bacto yeast extract (Difco). Plates were incubated for 72 h at 37°C in an atmosphere of 10% CO_2 in a gas incubator. Before inoculation, the shape and motility of the organisms were controlled by Gram staining and phase-contrast microscopy. MICs were determined by a modified broth dilution method, as previously described [21]. Briefly, twofold serial dilution of the test compounds were prepared in a 96-well microtiter plate containing 100 µL of MegaCellTM RPMI-1640 medium with 3% fetal calf serum (FCS). An inoculum equivalent to 1 McFarland standard was prepared in Wilkins Chalgren broth and diluted in MegaCellTM RPMI-1640 medium with 3% FCS. Each well was inoculated with *H. pylori* at a final concentration of approximately 5 x 10^5 CFU/well. The plates were

incubated at 37°C under microaerophilic conditions (10% CO_2 in a gas incubator) and examined after 72 h of incubation.

Acknowledgments - The authors are grateful to the Ministry of Science (Grant Nos. 173021 and 34012) for financial support.

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