原　著

Helicobacter pylori のハーブ走性及びハーブ抽出液による行動への影響

Herbtaxis of *Helicobacter pylori* and effects of herbs on its behavior.

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【Abstract】

Herbs have been used as conservative substances, appetite stimulating agents or relaxing materials in the history of food culture as a right herb in the right country. The effect of herbs on chemotactic activity of *Helicobacter pylori (H.pylori)* has not been investigated previously. We performed in vitro influence of herb extracts on growth, motility and chemotaxis of *H. pylori*. Species of herb specific inhibition on growth of *H. pylori* was observed. Lavender, Rosemary Stevia, Bergamot and Sage showed strong swarm inhibition. However, chemotaxis to herb extracts (herbtaxis) was observed on Yellow Flower Lavender, Stevia and Cherry Sage. Inhibition of growth and swarming ability of *H. pylori* by herb extracts might work as protective materials against *H. pylori*-associated gastroduodenal disease *in vivo*. Further investigation will be needed to ensure that this effect would be observed in animal model.

List of abbreviations: *H. pylori : Helicobacter pylori,* DMSO: dimethyl sulfoxide,

Keywords: *H. pylori*, herb extracts, chemotaxis, motility inhibition, swarming

Introduction

Helicobacter pylori is a well known bacterium which induces a chronic inflammatory response in the stomach and causes gastritis, peptic ulcer disease, gastric adenocarcinoma [1,2)], and gastriclymphoma[3,4)]. This microorganism has a spiral body with unipolar sheathed flagella, which is well adapted for its motility in the viscous environment [5)].

Motility in the viscous environment is one of the essential factors for *H. pylori* to colonize in the mucus layer of the gastric epithelium, as it has been reported that high motility by flagella was a significant factor for colonizing ability of *H. pylori* in the piglets model[6)]. By the comparison of the motile ability in viscous environment between enteric bacterium, *Salmonella* or *Escherichia coli*, and *Helicobacter* species, *Helicobacter* species exhibits much higher motile[7)]. Therefore, motility in viscous environment seems necessary for both colonization and persistence of the bacteria in the gastric mucosa.

On the other hands, herbs are used in many ways all over the countries, in many cases, as traditionally observed custom. It might be a possibility that difference of the incidence ratio of gastric diseases by *H. pylori* infection between other advanced countries and Japan would be ascribed by the difference of the food custom. Several studies on the growth inhibition of *H. pylori* by herbs and capsaicin were reported[8,9)].

We investigate here focusing on that how herb extracts affect on the motile ability in viscous environment and

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Table 1　List of herb extracts used in this work.

No.	Herb	No.	Herb
1	Sweet　Basil	40	Lemon Balm
2	Darkopal　Basil	41	Dill
3	Lettuce　Basil	42	Fennel(Leaf)
4	Cinnamon　Basil	43	Wild Strawberry(leaf)
5	Licorice　Basil	44	Sweet Herb　MEXICAN
6	Purpule Russell Basil	45	Hop
7	Bush Basil	46	Stevia
8	Holy Basil	47	Nasturtium(Leaf)
9	Ruby Basil	48	Oregano
10	Lemon Basil	49	Sweet　Marjoram
11	Sweet Basil Seed	50	Common Mallow
12	Yellow Flower Lavender	51	Lamb's Ears
13	Summer Lavender	52	Echinacea
14	Hidcote Lavender	53	Chives
15	French Lavender	54	Chicory
16	French Stoechas Lavender	55	Chives
17	Spike Lavender	56	Common Soapwort)
18	English Lavender	57	Black Mallow
19	Apple Mint	58	Roman Chamomile
20	Water Mint	59	Chervil
21	eau de cologne Mint	60	Wild Rocket
22	Orange Mint	61	Rose Geranium
23	Cool Mint	62	Common Comfrey
24	Spear Mint	63	Russian Trragon
25	Pineapple Mint	64	Rhubarb
26	Bush Mint	65	Salad Burnet
27	Pepper Mint	66	Common Yarrow
28	Rosemary Tuscanablue	67	Garlic
29	Rosemary Wood	68	Echinacea (Flowers)
30	Rosemary　Dreamyblue	69	Echinacea (Stem)
31	Apple Eucalyptus	70	Echinacea (Leaf)
32	Lemon Eucalyptus	71	Echinacea (Root)
33	Pineapple Sage	72	Commom Mullein (Leaf)
34	Cherry Sage	73	Commom Mullein (Fruit)
35	Amethyst Sage	74	Bergamot (Flower)
36	Clary Sage	75	Bergamot (Leaf)
37	Lemongrass	76	Hyssop (Leaf)
38	Lemon Thyme	77	Hyssop (Flower)
39	Lemon Scented Verbena		

attract *H. pylori in vitro*, in addition of dose-dependent growth inhibition by herb extracts, which are very common in western advanced countries.

Materials and methods

Bacteria strains and growth conditions.

Helicobacter pylori ATCC43504 was used in this study. Bacteria was grown on brucella broth supplemented with 3 % horse serum (brucella-serum broth) or on agar plates with brucella-serum broth solidified with 1.5 % agar (brucella-serum agar) and incubated under microaerobic conditions (N_2, 85%; O_2, 5%; CO_2, 10%) at 37 ℃ .

Herb extracts preparation.

Herbs used in this study were listed in Table 1. 5.0 g of row leaves of each herb was homogenized in ethanol and filtered. The filtrates were adjusted to 50 ml. 10ml of ethanol extraction was evaporated at 40 ℃ under reduced

Table 2 Dose dependent growth inhibition of *H. pylori* by herb extracts.

	amount of row leaf (mg)	AVR (cm)		amount of row leaf (mg)	AVR (cm)
French Lavender	control	1.38	Cherry Sage	control	1.09
	40	2.80		40	3.89
	4	1.72		4	2.30
	0.8	1.43		0.8	1.42
	0.4	1.44		0.4	1.26
French Stoechas Lavender	control	1.28	Amethyst Sage	control	1.21
	40	3.42		40	3.18
	4	2.17		4	1.27
	0.8	1.61		0.8	1.18
	0.4	1.55		0.4	1.12
eau de cologne Mint	control	ND	Bergamot (Flower)	control	ND
	40	2.82		40	4.02
	4	1.81		4	2.64
	0.8	1.26		0.8	1.56
	0.4	0.98		0.4	1.16
Rosemary Tuscanablue	control	1.28	Bergamot (Leaf)	control	1.46
	40	3.35		40	4.78
	4	2.07		4	2.12
	0.8	1.63		0.8	1.44
	0.4	1.52		0.4	1.39
Rosemary Dreamyblue	control	1.20	Hyssop (Flower)	control	ND
	40	3.07		40	5.43
	4	2.26		4	2.26
	0.8	1.81		0.8	1.25
	0.4	1.67		0.4	1.17
Lemon Thyme	control	1.03	Common Soapwort)	control	ND
	40	3.13		40	3.58
	4	1.64		4	1.45
	0.8	1.58		0.8	1.14
	0.4	1.19		0.4	1.06
Stevia	control	1.29	Roman Chamomile	control	ND
	40	3.12		40	4.95
	4	2.40		4	2.32
	0.8	1.91		0.8	1.51
	0.4	1.53		0.4	1.35
Pineapple Sage	control	1.15	Common Yarrow	control	ND
	40	3.65		40	3.32
	4	1.55		4	1.83
	0.8	1.16		0.8	1.31
	0.4	1.06		0.4	1.13

pressure. The residues were dissolved in 500 µl of DMSO and used as herb extracts for further experiments.

Bacterial growth inhibition studies.

0.5 ml of 2 days culture of *H. pylori* cells were added to the 2.0 ml of brucella-serum broth containing 0.5 % refined agar (brucella soft agar) and poured onto brucella-serum agar. The 8mm-diameter filter paper disk (Toyo Roshi, Tokyo Japan) exudated 20 µl of herb extracts were placed onto the brucella soft agar. Herb extracts were diluted with DMSO for the dose dependent inhibition. Plates were incubated under microaerobic condition for 3days at 37 ℃. The diameter of clear zone was measured. Data are expressed as the means and standard errors for triplicate experiments.

Motility assay.

H. pylori (2 X 10^6 cells) were stabbed with toothpick onto a motility agar containing 0.3 % refined agar in brucella-serum broth. 10 µl of herb extract (equivalent with 20 mg of row leaves) was premixed to the motility agar. Plates were incubated for 5 days in microaerobic condition at 37 ℃. Data were expressed as the means and standard errors for triplicate experiments.

Chemotactic response to herb extracts.

Cells grown in brucella-serum broth were collected and washed twice with 50 mM potassium phosphate buffer, pH7.0 (chemotaxis buffer) and suspended in chemotaxis buffer. 10^{-3} diluted herb extracts were filled in 1 µl capillaries and performed to the chemotaxis assay to herb extract (herbtaxis) described previously[10]. After incubation for 5, 10 and 30 min, bacteria incorporated into the capillary tube were spread over a 0.125 cm^2 area on a slide glass, Gram stained and counted. Data was expressed as the means and standard errors for three determinations.

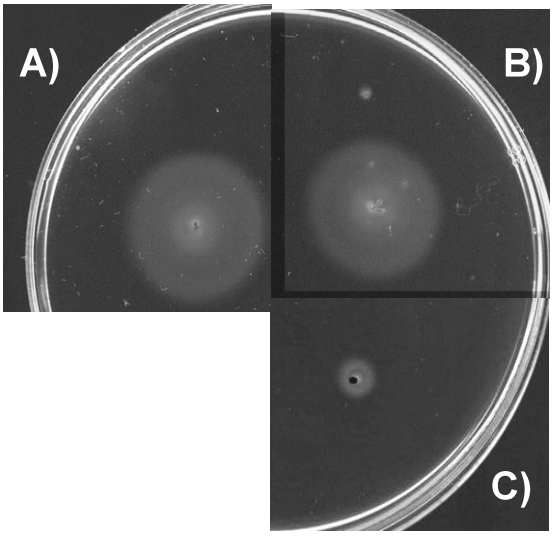

Fig.1. Motility testing of *H. pylori*. Single colony of *H. pylori* ATCC43504 was stabbed onto motility agar plate with DMSO (A), French Stoechas Lavender extract (B) and Bergamot extract (C). The sizes of haloe were measured after 5 days incubation.

Results

Growth inhibition of *H. pylori* by herb extracts.

First screenings of the growth inhibition of *H. pylori* by 77 kinds of herb extracts were tested (data not shown). 16 herb extracts were selected to examine a dose-dependent manner of growth inhibition at a corresponding concentration of 0.4 mg raw leaf ml^{-1} (Table 2). The inhibitory effect was observed in several herbs associated with the biological classification as overall view. For example, Basil spp. and Mint spp. has weak inhibition by the comparison with Lavender, Rosemary and Sage. Remarkable dose-dependent growth inhibition was observed in16 herb extracts as shown in Table 3. Interestingly though DMSO did not affect growth of the bacteria, clear growth inhibition circle was observed around the disk of DMSO which was placed as a control disk of each herb extract on the several agar plates, such as French Lavender, French Stoechas Lavender, Rosemary Tuscanable, Rosemary Dreamyblue, Lemon Thyme, Stevia, Pineapple Sage, Amethyst Sage, Cherry Sage, Roman Chamomile and Bergamot.

Swarming inhibition of *H. pylori* by herb extracts.

15 herbs were selected to test the swarming inhibition

Table 3　Effect of herb extracts to swarming ability of *H. pylori*

	AVR(cm)	±SD
Yellow Flower Lavender	0.61	0.17
Hidcote Lavender	1.38	0.42
French Lavender	1.46	0.30
French Stoechas Lavender	1.24	0.10
Rosemary Tuscanablue	1.37	0.26
Rosemary Wood	0.57	0.49
Rosemary Dreamyblue	0.65	0.40
Cherry Sage	0.12	0.05
Amethyst Sage	0.98	0.10
Roman Chamomile	1.67	0.51
Common Yarrow	1.39	0.29
Lemon Thyme	1.32	0.35
Stevia	0.12	0.16
Bergamot (Flower)	1.05	0.30
Bergamot (Leaf)	0.63	0.09
Brucella agar	1.86	0.34
DMSO 10µℓ	1.59	0.32

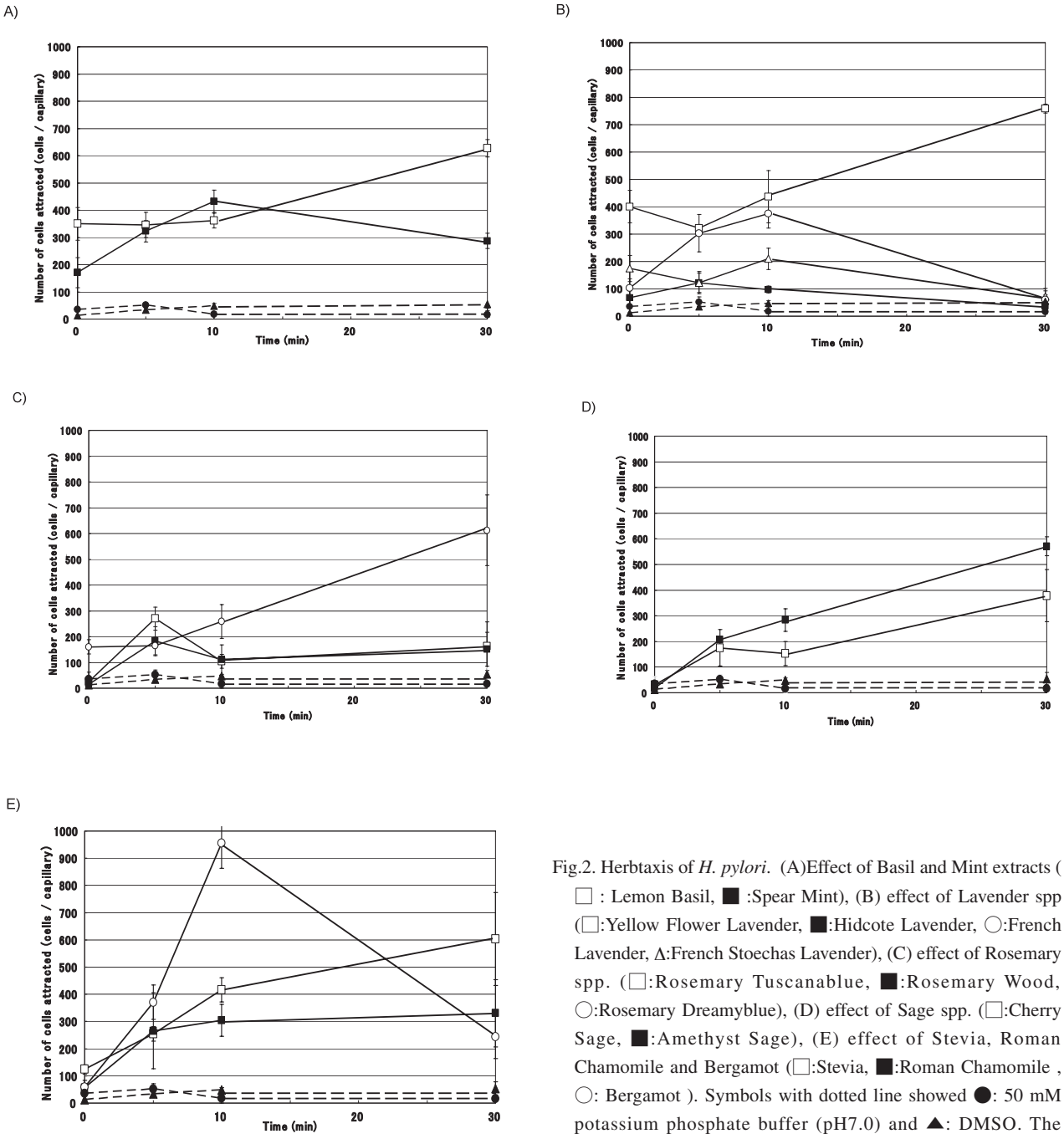

Fig.2. Herbtaxis of *H. pylori*. (A)Effect of Basil and Mint extracts (□ : Lemon Basil, ■ :Spear Mint), (B) effect of Lavender spp (□:Yellow Flower Lavender, ■:Hidcote Lavender, ○:French Lavender, Δ:French Stoechas Lavender), (C) effect of Rosemary spp. (□:Rosemary Tuscanablue, ■:Rosemary Wood, ○:Rosemary Dreamyblue), (D) effect of Sage spp. (□:Cherry Sage, ■:Amethyst Sage), (E) effect of Stevia, Roman Chamomile and Bergamot (□:Stevia, ■:Roman Chamomile , ○: Bergamot). Symbols with dotted line showed ●: 50 mM potassium phosphate buffer (pH7.0) and ▲: DMSO. The number of cells attracted by herb extract in the capillary was counted. Error bars show standard deviations.

efficacy from the growth inhibition data. The concentric circle of the swarm zone shown in Fig. 1 for the example was observed on the motility plate. The motility plate containing 10 µl of DMSO did not affect any swarm inhibition of *H. pylori*. However, Yellow Flower Lavender, Rosemary Wood, Rosemary Dreamyblue, Cherry Sage, Stevia and Bergamot (leaf) showed strong swarm inhibition (Table 3).

Chemotaxis to herb extracts.

Herbs which did not show growth inhibition of *H. pylori*, Basil spp. and Mint spp., acted as attractant to *H. pylori* (Fig. 2 (A)). As shown in Fig.2 (B), most herbtaxis to Lavender spp.of *H. pylori* was low, however, Yellow Flower Lavender showed high activity as an attractant. Rosemary Dreamyblue highly attracted *H. pylori* among Rosemary spp.(Fig.2 (C)). Amethyst Sage

showed the highest activity as an attractant among Sage spp(Fig. 2 (D)). Stevia, Roman Chamomile and Bergamot showed positive activity to attract *H. pylori* though the number of cells accumulated in the capillary was reduced after 10 min incubation in case of Stevia (Fig. 2 (E)).

Discussion

H. pylori has a small genome of only 1.7 Mb[11] in size. However, detailed investigation of the genome structure of different isolates showed that this organism has a diverse genome[12], which was explained by the high occurrence of natural transformation in this organism[13]. Investigation of factors influencing eradication of *H. pylori* with triple therapy[14] showed that it is subject to failure related to bacterial resistance to the antibiotics[15].

Our results demonstrate that growth inhibition of *H. pylori* in vitro inclined towards genus specific efficacy. For example, in the same family of Labiatae, Basil and Mint groups did not show strong growth inhibition in comparison with that shown by Lavenders and Sages. The major compounds which are contained in the former groups are methyl carbitol (Basils) and menthol derivatives (Mints). Major components in the latter groups are limonene, linalyl acetate, cineol and pinene. Linalool is contained in both groups. Each compound was tested for the growth inhibition. α-pinene, β-pinene and 1.8-cineol showed quite strong growth inhibition over 2.5 μmol, 5 μmol and 5 μmol respectively. However, other compounds but linalool did not show typical growth inhibition. Minimum molecule of linalool for growth inhibition of *H. pylori* was 25 μmol. However, the active compounds which were contained in these herb extracts have not yet been determined. The growth inhibition observed on control disks in experiments using extracts of Lavenders and Sages indicated that these herb extracts contained volatile, DMSO-soluble compounds which acted as inhibitory factors, though the identity of these compounds is not known.

Motility assays in soft agar using extracts of Yellow Flower Lavender, Rosemary Wood, Rosemary Dreamyblue, Stevia, Bergamot (leaf) and Cherry Sage all showed reduction in motility relative to control experiments. However, Rosemary Dreamyblue, Stevia, Bergamot (leaf) and Cherry Sage all displayed strong inhibition of growth so in these cases the results may reflect lack of growth rather than inhibition of motility. The motility inhibition observed using Yellow Flower Lavender and Rosemary Wood suggests that these herb extracts might inhibit *H. pylori* movement in the mucin layer in the stomach.

H. pylori showed a chemotactic attraction toward extracts of Yellow Flower Lavender, Stevia, Rosemary Dreamyblue, Bergamot and Amethyst Sage suggesting these herbs may be useful in eliciting *H. pylori* from the basement toward the surface of the gastric mucosa. In the cases of Bergamot and Stevia the number of cells accumulated in capillaries was decreased after ten minutes incubation, suggesting the extracts contain compounds with a toxic effect on *H. pylori*, consistent with the strong growth inhibition observed with these extracts. Herb extracts which work both as growth inhibitors and as attractants, such as rosemary Dreamyblue, Amethyst Sage and Bergamot, may effectively elicit *H. pylori*.

From the genome annotation data, *H. pylori* has only 4 transducer-like protein coding regions[11), 12], though substrates for their proteins are still unknown. However, the active compounds in herb extracts which attracted *H. pylori* might have a similar three-dimensional molecular structure to the substrates for the sensors. The investigation of the response of *H. pylori*, and of *H. pylori* mutants in which transducer-coding genes have been disrupted, to concentration gradients of herb extracts will be needed to obtain further information.

The data presented here suggests that the proper usage of herbs may help to decrease gastric disease caused by *H. pylori* infection. Appropriate use of herbs in daily foods might be preferable to medical treatment with the risks of side effects or causing the appearance of antibiotic resistant strains of *H. pylori*. This effect deserves further study in animal models.

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