

## Helicobacter pylori および Streptococcus mutans に対するシソ科ハーブ4種の抽出液の in vitro 抗菌活性

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# In Vitro Antibacterial Activity of Extracts from Four *Labiatae* Herbs Against *Helicobacter pylori* and *Streptococcus mutans*<sup>†</sup>

Keiko AZUMA, Hidekazu ITO, Katsunari IPPUSHI and Hisao HIGASHIO\*

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## Synopsis

In vitro antibacterial activity of extracts from four *Labiatae* herbs, perilla (*Perilla ocymoides* L.), peppermint (*Mentha piperita* L.), sage (*Salvia officinalis* L.) and thyme (*Thymus vulgaris* L.) against two human pathogens *Helicobacter pylori* and *Streptococcus mutans* was evaluated. The highest activity against both bacteria was observed in sage extract, followed by perilla and thyme extracts. Their major volatile components were identified as perillaldehyde and limonene (perilla extract), menthol (peppermint extract),  $\alpha$ -thujone and camphor (sage extract) and thymol (thyme extract) by GC and GC-MS. The antibacterial activity of the major volatile components against *H. pylori* and *S. mutans* was investigated and its relation to the activity of the four plant extracts was discussed.

**Key Words:** *Helicobacter pylori*, *Streptococcus mutans*, antibacterial activity, *Labiatae*, volatile components

## I Introduction

The physiological functionality of plant foods including vegetables and fruits has received much attention and has been studied in vitro and in vivo by many researchers (OHIGASHI et al., 1996; AZUMA et al., 1999; YAMAZAKI, 1999). In particular, herbs are important not only for their use as flavorings but also for their biological activities such as anti-microbial (MARUZZELLA and LIGUORI, 1958; UEDA et al., 1982; ZAIKA, 1988), anti-oxidative (NAKATANI, 1993), anti-inflammatory and anti-allergic (IPPOUSHI, 2000; UEDA and YAMAZAKI, 2001).

Gastric infection with *Helicobacter pylori* has been shown to cause gastric and peptic ulcers and enhance the risk of gastric cancer (MARSHALL et al., 1985; CORREA, 1997). In fact, its infection ratios are considerably high in developed countries where there is a high prevalence of gastric cancer. Thus, the eradication of *H. pylori* is important for the prevention of gastroduodenal diseases in infected people and treatment of patients with these diseases. In recent years, the in vitro antibacterial activity of some plants, including herbal medicines, against *H. pylori* has been found, and some compounds, such as capsaicin, thiosulfinate, decursinol angelate, decursin, anacardic acids and (*E*)-2-hexanal, have been isolated as their active components (FABRY, et al., 1996; INGOLFSDOTTIR et al., 1997; JONES et al., 1997; BAE, et al., 1998; KUBO et al., 1999; BAE, et al., 1999). Recently, FAHEY et al. (2002) has reported that a kind of isothiocyanates, named sulforaphane, in broccoli is highly effective in inhibiting *H. pylori* infections and blocking gastric tumor formation.

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Department of Physiology and Quality Science  
360 Kusawa, Ano, Mie, 514-2392 Japan

\* Department of Leaf and Root Vegetables

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Dental caries are also ubiquitous diseases in developed regions. It is known that the diseases are caused by an increase in strongly acidogenic gram-positive bacteria such as *Streptococcus mutans*. Therefore, theoretically, dental caries can be prevented by eliminating *S. mutans*, the primary cariogenic bacterium. In previous searches for naturally occurring antibacterial agents against this bacterium, it was reported that plant foods such as green tea, onion, roasted coffee and cacao bean husk or their components exhibited antibacterial activities against *S. mutans* (SAKANAKA et al., 1989; KUBO et al., 1992; MUROI and KUBO, 1993; KIM, 1997; DAGLIA et al., 1998; ZICHY and NOVAK, 1998; DEL CAMPO et al., 2000; OSAWA et al., 2001).

However, many aromatic plants potentially useful for the inhibition of *H. pylori* and *S. mutans* have not been investigated closely. This study aimed to evaluate in vitro antibacterial activity of extracts from four herbs of the *Labiatae* family against *H. pylori* and *S. mutans*, to show the possibility of their application for the prevention of gastroduodenal diseases and dental caries in humans.

## II Materials and Methods

### 1. Plant materials

Perilla (*Perilla ocymoides* L.), peppermint (*Mentha piperita* L.), sage (*Salvia officinalis* L.) and thyme (*Thymus vulgaris* L.) (Takii Seed Co. Ltd) were cultivated in the field of the National Institute of Vegetable and Tea Science in 1996. Immediately after harvesting, the leaves of these plants were washed with tap water and freeze-dried.

### 2. Chemicals and media

Camphor,  $\rho$ -cymene, linalool, perillaldehyde,  $\alpha$ -pinene,  $\gamma$ -terpinene and thujone were purchased from Tokyo Kasei Organic Chemicals Co. (Tokyo, Japan). Carvacrol, 1,8-cineole, L-(-)-limonene and thymol were obtained from Wako Pure Chemicals Co. (Osaka, Japan), and L-menthol was from Nacalai Tesque Co. (Kyoto, Japan). Blood agar base No.2 (CM 271) and *H. pylori* selective supplement SR147E were obtained from Oxoid Ltd. (Basingstoke, UK). Trypticase soy broth was purchased from Becton Dickinson (Cockeysville, MD), and yeast extract was from Difco Lab. (Detroit, MI). Defibrinated horse blood was obtained from Kohjin-Bio Co. (Saitama, Japan).

### 3. Preparation of plant extracts

Thirty grams of freeze-dried plant materials were immersed into one liter of diethylether and kept at room temperature overnight. The solvent was evaporated *in vacuo*. The residues were dissolved in 15 ml of 80% ethanol. After centrifugation for 5 min at 1000g, the supernatants were filtered through a membrane filter (DISMIC-25CS, 45  $\mu$ m). The plant extracts were kept at  $-40^{\circ}\text{C}$  until analysis or assay.

### 4. Identification and quantitative analysis of volatile components

Volatile components of the plant extracts were identified and quantitatively analyzed by gas chromatography (GC) and gas chromatography - mass spectrometry (GC-MS). The GC analysis was performed using a SHIMADZU GC-14A under the following conditions: column: DB-WAX (J&W Scientific, Folsom, CA), 60 m  $\times$  0.315 mm i.d., 0.50  $\mu$ m, carrier: He 1.0ml/min, column temperature: 70 $^{\circ}\text{C}$  for 10 min—220 $^{\circ}\text{C}$  at 4 $^{\circ}\text{C}$ /min, injector: split (1: 50), 250 $^{\circ}\text{C}$ , detector: FID, 250 $^{\circ}\text{C}$ . The quantitative data were obtained by an external standard method. Volatile components were identified using a HITACHI M-2030 gas chromatograph - M-2000S Mass Spectrometer set at an ionization voltage of 70 eV, under the same GC conditions mentioned above.

## 5. Bacterial strains

The test strain *H. pylori* ATCC 43504 was purchased from American Type Culture Collection (Rockville, MD). The test strain *S. mutans* IFO 13955 was obtained from Institute for Fermentation, Osaka (IFO, Osaka).

## 6. Antibacterial assay

The minimum inhibitory concentration (MIC) of the plant extracts and test compounds (Table 3) against *H. pylori* and *S. mutans* was measured as follows. Serial 2-fold dilutions of the plant extracts and test compounds were prepared as 80% ethanol solutions. The highest concentration of tested compounds was 4.0 mM.

### 1) *H. pylori*

Blood agar base was sterilized for 15 min at 121°C, then selective supplement and 7% horse blood were added. The agar plate consisted of the culture medium (4.9 ml) and the test solution (0.1 ml) was inoculated with *H. pylori* by a streak and incubated microaerobically for 3 days at 37°C in an anaerobic jar with BBL CampyloPak Plus (Becton Dickinson, Cockeysville, MD).

### 2) *S. mutans*

Each dilution of the extracts or compounds (0.1 ml) was aseptically added into 4.9 ml of autoclaved trypticase soy broth containing 0.5% yeast extract. The broth was then supplemented with 10  $\mu$ l of diluted inoculum containing approximately 10<sup>6</sup> cfu/ml *S. mutans*. The cultures were incubated stationary for 24 h at 37°C.

For each bacterium, a culture growth control without extract or compound (solvent only) was treated in the same way. All experiments were conducted in triplicate. The MIC was defined as the lowest concentration of extract or compound that prevented visible bacterium growth.

## III Results

### 1. Plant extract components

The volatile components of perilla, peppermint, sage and thyme extracts were analyzed by GC and GC-MS. Fig. 1 shows a GC chromatogram of the sage extract. The analyses of the other three extracts also exhibited more than 30 peaks. Among them, the major volatile components were identified and quantitatively determined as summarized in Table 1, compounds being listed in order of their elution time on the DB-WAX column. The perilla extract contained large amounts of perillaldehyde (69.7 mM) and limonene (50.9 mM) and relatively low amounts of some compounds such as  $\beta$ -caryophyllene and farnesene. The most abundant volatile component of the peppermint extract was menthol (71.3 mM) followed by 1,8-cineole (17.7 mM) and limonene (15.2 mM). The sage extract contained  $\alpha$ -thujone (110.9 mM) and camphor (70.8 mM) as the primary volatile components, and several other compounds including 1,8-cineole and  $\alpha$ -pinene. The largest component of the thyme extract was thymol; its content of 166.4 mM was remarkably high compared with those of other components such as  $\rho$ -cymene (45.5 mM) and  $\gamma$ -terpinene (44.2 mM).

### 2. Antibacterial activity of plant extracts against *H. pylori* and *S. mutans*

The sensitivity of *H. pylori* and *S. mutans* to perilla, peppermint, sage and thyme was evaluated by means of agar plates or broth with different concentrations up to 2.0% of their extracts. The MICs against *H. pylori* and *S. mutans* are shown in Table 2. All four plant extracts exhibited some degree of antibacterial activity against these bacteria, with the exception of the peppermint extract against *S. mutans* which showed no activity under the experimental conditions used in this study. Among the four tested extracts, the sage extract showed the highest activity against both bacteria, with a MIC of 0.125% (v/v). The perilla and thyme extracts appeared to

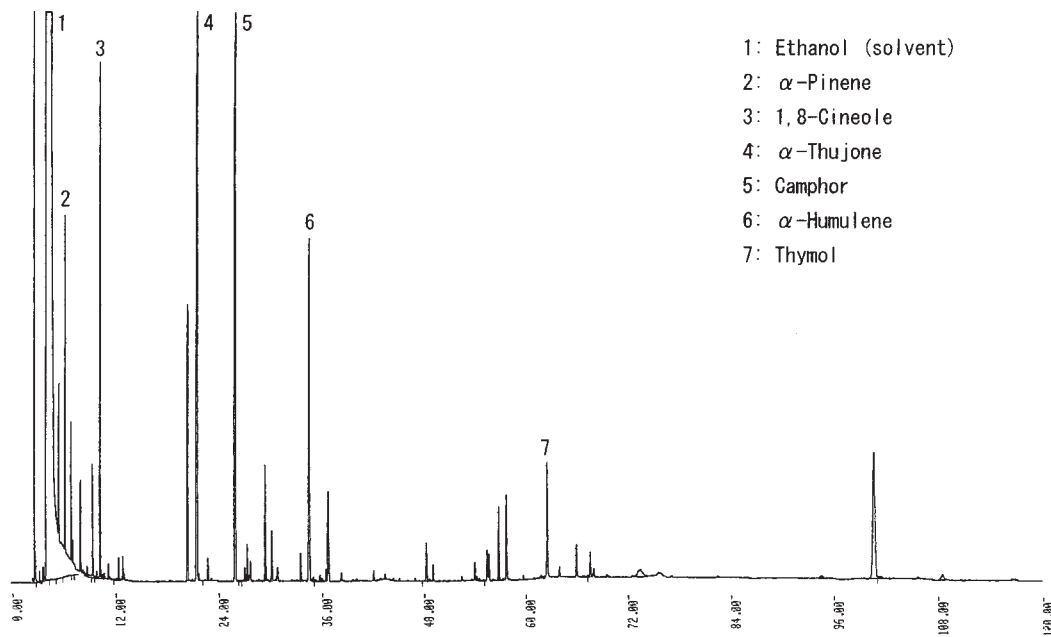


Fig. 1 GC chromatogram of the sage extract.

Table 1 Major volatile components and their contents of four *Labiatae* herb extracts

Plant	Volatile component	Content (mM)*
Perilla	Limonene	50.9 $\pm$ 2.7
	Linalool	2.8 $\pm$ 0.2
	$\beta$ -Caryophyllene	16.5 $\pm$ 0.5
	Farnesene	13.4 $\pm$ 0.4
	Perillaldehyde	69.7 $\pm$ 3.1
	Perillyl alcohol	7.4 $\pm$ 0.4
Peppermint	$\alpha$ -Pinene	6.9 $\pm$ 0.3
	Limonene	15.2 $\pm$ 0.9
	1,8-Cineole	17.7 $\pm$ 0.8
	Menthone	2.7 $\pm$ 0.2
	Menthofuran	3.4 $\pm$ 0.2
	Menthol	71.3 $\pm$ 2.9
Sage	$\alpha$ -Pinene	17.0 $\pm$ 0.5
	1,8-Cineole	19.6 $\pm$ 0.8
	$\alpha$ -Thujone	110.9 $\pm$ 4.8
	Camphor	70.8 $\pm$ 3.0
	Humulene	12.1 $\pm$ 0.4
	Thymol	8.3 $\pm$ 0.4
Thyme	$\alpha$ -Pinene	15.5 $\pm$ 0.5
	$\alpha$ -Terpinene	13.4 $\pm$ 0.3
	$\gamma$ -Terpinene	44.2 $\pm$ 1.9
	$\rho$ -Cymene	45.5 $\pm$ 2.1
	Linalool	3.2 $\pm$ 0.2
	Carvacrol	12.7 $\pm$ 0.4

\* GC analysis was repeated three times per each extract.  
 Values are mean  $\pm$ SD of three replications.

Table 2 Antibacterial activity of four *Labiatae* herb extracts against *Helicobacter pylori* and *Streptococcus mutans*

Plant	MIC(% (v/v))	
	<i>H. pylori</i>	<i>S. mutans</i>
Perilla	0.25	0.25
Peppermint	0.50	>2.00
Sage	0.125	0.125
Thyme	0.25	0.25

MIC: Minimum inhibitory concentration

Table 3 Antibacterial activity of selected volatile compounds against *Helicobacter pylori* and *Streptococcus mutans*

Compound	MIC (mM)	
	<i>H. pylori</i>	<i>S. mutans</i>
Camphor	2.00	4.00
Carvacrol	0.50	1.00
1,8-Cineole	>4.00	1.00
$\rho$ -Cymene	>4.00	0.25
L-Limonene	>4.00	0.125
Linalool	1.00	4.00
L-Menthol	0.50	0.50
Perillaldehyde	0.50	4.00
$\alpha$ -Pinene	>4.00	0.50
$\gamma$ -Terpinene	>4.00	0.25
$\alpha$ -Thujone	2.00	2.00
Thymol	0.50	1.00

MIC: Minimum inhibitory concentration

be equally effective against both *H. pylori* and *S. mutans*; their MIC value was 0.25% (v/v). The peppermint extract was found to be the least active against both bacteria.

### 3. Antibacterial activity of volatile components against *H. pylori* and *S. mutans*

Among the plant extract components identified, twelve compounds shown in Fig. 2 were selected based on their contents (Table 1) and reported antibacterial properties (IWAI and NAKATANI, 1989). They were evaluated for their inhibitory activity against *H. pylori* and *S. mutans*. As summarized in Table 3, carvacrol, L-menthol, perillaldehyde and thymol, with a MIC of 0.5mM, exhibited the highest activity against *H. pylori*, and linalool, with a MIC of 1.0 mM, followed them. On the other hand, 1,8-cineole,  $\rho$ -cymene, L-limonene,  $\alpha$ -pinene and  $\gamma$ -terpinene could not inhibit the growth of *H. pylori*, even at the highest concentration of 4.0 mM.

All the tested compounds demonstrated various degrees of antibacterial activity against *S. mutans* (Table 3). The most active compound was found to be L-limonene, with a MIC of 0.125 mM, followed by  $\rho$ -cymene and  $\gamma$ -terpinene, with a MIC of 0.25 mM. Camphor, linalool and perillaldehyde were the least effective for the growth inhibition of this bacterium.

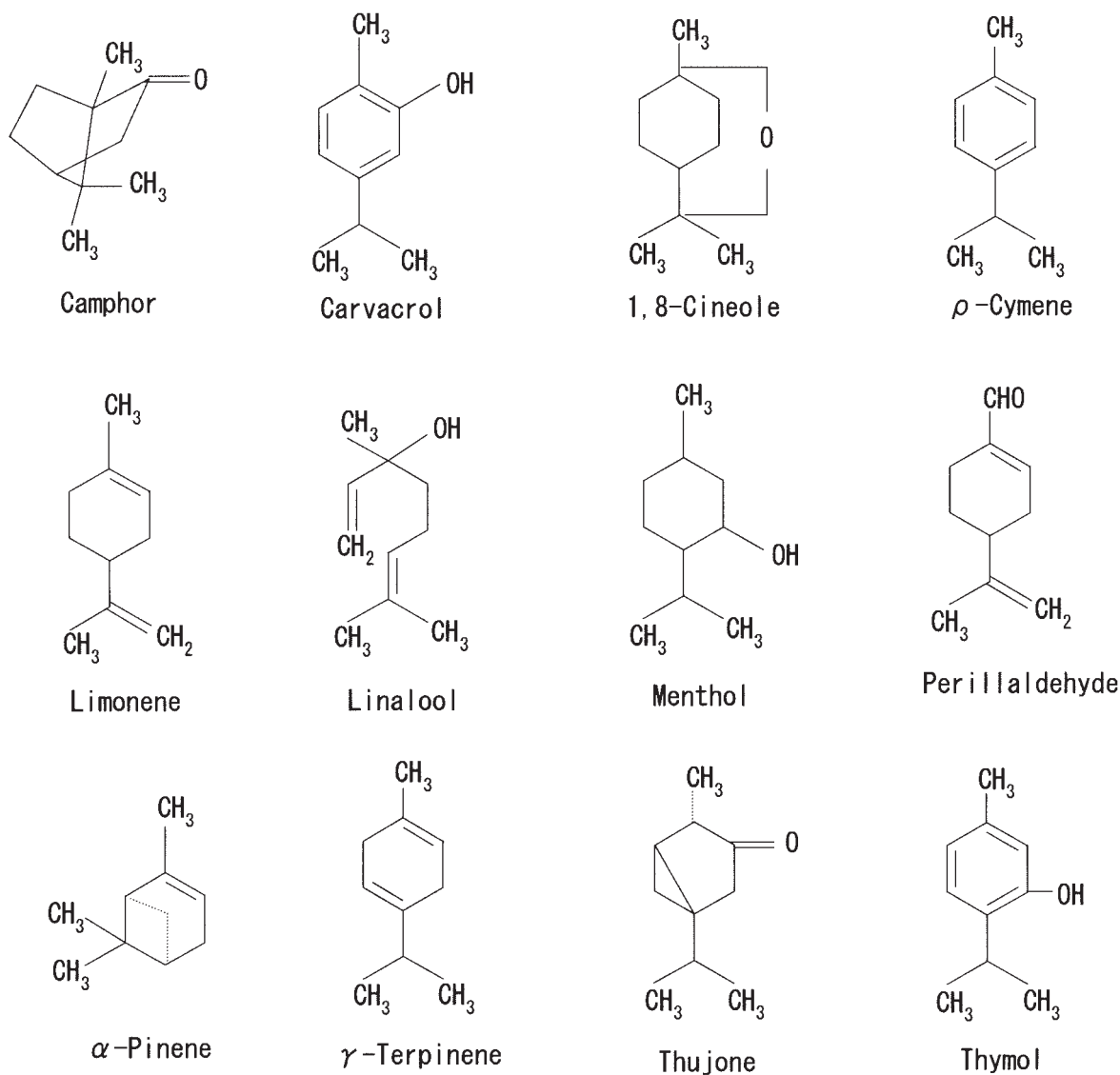


Fig.2 Chemical structures of tested volatile compounds.

## IV Discussion

Aromatic plants have been known and valued for their preservative qualities, as well as for their flavors. Previously, many researchers have reported the antifungal and antibacterial activity of essential oils or extracts of aromatic plants against food poisoning or spoilage microorganisms and animal or plant pathogens (SHELEF, 1983; UEDA et al, 1982; SHELEF et al., 1984; LAURA et al., 1988; IWAI and NAKATANI, 1989; HUSSEIN, 1990; SHAPIRO et al. 1994; SIVROPOULON et al., 1995; OUATTARA et al., 1997; DORMAN et al., 2000). However, there are few previous reports on the comparison of the antibacterial activity of some *Labiatae* herbs, including perilla (INOUE et al., 2001). In the present study, perilla appeared to exhibit activity equal to thyme and higher than peppermint against both *H. pylori* and *S. mutans* (Table 2). It had been reported that herbs were, in general, more effective against gram-positive bacteria than gram-negative bacteria (UEDA et al., 1982). It is noted that the sensitivity of the gram-negative bacterium *H. pylori* to the four *Labiatae* herbs was almost the same or higher compared with that of the gram-positive bacterium *S. mutans* in this study.

Since it is known that many of the antimicrobial components of herbs are present in their essential oils (UEDA et al., 1982), we analyzed the volatile components of perilla, peppermint, sage and thyme extracts, prepared in this study. The major volatile constituents identified from each plant (Table 1) were in agreement with the previous reports on the essential oils of perilla (ITO, 1970; WATANABE et al., 2000), peppermint (MAROTTI et al, 1994; MORI et al, 2002), sage (IWAI and NAKATANI, 1989; GUILLEN and CABO, 1996) and thyme (IWAI and NAKATANI, 1989; SENATORE, 1996), although there might be some differences in their composition.

Each plant extract, except for peppermint extract, was equally active against *H. pylori* and *S. mutans* (Table 2), whereas the MICs of the tested volatile components against *H. pylori* did not always agree with those against *S. mutans* (Table 3). Many of the tested compounds were likely to show lower MICs against *H. pylori* than against representative food bacteria (AZUMA et al., 1999).

The compounds with phenolic structures, such as carvacrol and thymol, possessed powerful antibacterial activity against the test bacteria, in particular, *H. pylori*. These results are in agreement with published data on their activity against animal or plant pathogens and food bacteria (IWAI and NAKATANI, 1989; SHAPIRO et al., 1994; LIS-BALCHIN and DEANS, 1997; DORMAN and DEANS, 2000). DORMAN and DEANS (2000) confirmed the importance of the hydroxyl group in the phenolic structure in antibacterial activity, and also found that the relative position of the hydroxyl group exerted an influence on the activity. In this study, however, we observed no differences in the activity between carvacrol and thymol, which are different in their relative position of the hydroxyl group.

Monoterpene cyclic hydrocarbons  $\rho$ -cymene,  $\alpha$ -pinene and  $\gamma$ -terpinene, without phenolic structures, are not known as powerful antibacterial agents. It should be noted that these compounds were highly active against *S. mutans*, despite the lack of activity against *H. pylori*. Their action mechanisms on *S. mutans* should be clarified in further studies.

Among the tested monoterpene alcohols, L-menthol appeared to be significantly more active against *S. mutans* compared with linalool. It is supposed that  $\rho$ -menthane skeleton included in L-menthol may be related to its activity. Monoterpene ketones, camphor and thujone, were likely to be less active than the tested monoterpene alcohols. These results were in agreement with their activities against different microorganisms reported by UEDA et al. (1982). Interestingly, perillaldehyde, the component characteristic to perilla, appeared to be preferentially much more effective for growth inhibition of *H. pylori*, in contrast with monoterpene hydrocarbons such as  $\rho$ -cymene,  $\alpha$ -pinene and  $\gamma$ -terpinene. Its powerful activity might be explained by a highly electronegative arrangement formed by an aldehyde group conjugated to a carbon double bond, as

suggested for the high antimicrobial activity of aldehydes (MOLEYER and NARASIMHAM, 1986).

It is expected that these volatile components might contribute to the antibacterial activity of the tested *Labiatae* herb extracts. From the results presented in Tables 1-3, the major component responsible for the activity of the perilla extract against *H. pylori* is thought to be perillaldehyde, and against *S. mutans* it might be limonene. The activity of the peppermint extract against *H. pylori* is probably due to menthol, its primary constituent. Although the broth added with 2% (v/v) of the peppermint extract contained menthol and limonene at higher concentrations than their MICs against *S. mutans*, it could not inhibit the growth of this bacterium. It is supposed that some constituents present in the peppermint extract might interfere with and weaken the antibacterial action of menthol and limonene. Thymol, found in large amounts in the thyme extract, is expected to greatly contribute to its activity against *H. pylori*, while some components including  $\rho$ -cymene and  $\gamma$ -terpinene as well as thymol might be related to its activity against *S. mutans*.

On the other hand, the remarkable activity of the sage extract could not be explained by the main volatile components found in the extract. It is supposed that unidentified volatile compounds observed on the GC chromatogram in Fig. 1 and/or non-volatile components such as phenolic compounds with abietatriene or related structure (GUILLEN and MANZANOS, 1999) could be responsible for the activity of the sage extract. There is also a possibility that these components may interact synergistically to its activity.

This in vitro study has suggested that *Labiatae* herbs such as perilla, sage and thyme can contribute to the eradication of *H. pylori* and *S. mutans* for the prevention of gastroduodenal diseases and dental caries in vivo. Further investigations are needed to make clear the ingestion amounts of these herbs required for the prevention of these diseases in humans.

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## *Helicobacter pylori* および *Streptococcus mutans* に対する シソ科ハーブ 4 種の抽出液の in vitro 抗菌活性

東 敬子・伊藤 秀和・一法師 克成・東尾 久雄\*

### 摘 要

4 種のシソ科ハーブ、アオシソ、ペパーミント、セージ、およびタイムの抽出物の *Helicobacter pylori* および *Streptococcus mutans* に対する抗菌活性を in vitro で評価した。セージ抽出液はいずれの菌に対しても最も高い抗菌活性を示し、次いでアオシソとタイムの抽出液の活性が高かった。それらの抽出液の主要な揮発性成分として、アオシソにはペリラルデヒド、リモネン、ペパーミントにはメントール、セージには $\alpha$ -ツヨン、カンファー、タイムにはチモールなどが認められた。それら成分のうち、*H. pylori* に対して最も高い活性を示したのはカルバクロール、メントール、ペリラルデヒド、チモール（最小生育阻止濃度 0.5 mM）であった。*S. mutans* に対しては L-リモネンが最も高い活性（最小生育阻止濃度 0.125 mM）を示し、次いで $\rho$ -シメンと $\gamma$ -テルピネンの活性が高かった。アオシソ、ペパーミント、タイムの抽出液の抗菌活性は主要な揮発性成分によるものであることが示唆されたが、セージ抽出液の強い抗菌活性には未同定の揮発性成分や不揮発性成分も寄与しているものと考えられた。