

# Antibacterial activity of *Hypericum erectum*

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**Running title:** Antibacterial activity of *H. erectum*

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**Abstract:**

In recent years, the potential of oral care in preventing aspiration pneumonia has been recognized. Consuming drinks is thought to be an easy and effective method of oral care, and the antibacterial activities of various drinks have been examined. However, side effects associated with, for example, caffeine as an ingredient in tea (e.g. sleep disorders) need to be taken into consideration. As yet, a safe caffeine-free tea to be taken orally to prevent aspiration pneumonia has not been reported. Thus, in the present study we evaluated the antibacterial effects of hot water extracts of four teas, namely *Hypericum erectum*, *Crataegus cuneata*, *Rosa canina*, and *Matricaria rectita*, thought to be caffeine-free. The effects of the extracts against 19 bacteria and 1 fungus were investigated by the dilution plate technique. In addition, the components of the teas were analyzed by HPLC analysis. The strongest antibacterial activity was observed for the hot water extract of *H. erectum*, which exhibited significant activity against oral bacteria, including *Streptococcus oralis*. However, the *H. erectum* extract did not kill microbiota, such as *Escherichia coli* and *Lactobacillus casei*. Neither hypericin nor caffeine, both of which have notable side effects, were

detected in the *H. erectum* extract following HPLC analysis. These results suggest that *H. erectum* tea may be a good candidate for simple, safe oral care to prevent aspiration pneumonia in the elderly.

**Keywords:** oral care, *Hypericum erectum*, caffeine, hypericin, hot water extract

## Introduction

In recent years, it has been demonstrated that mouth care is important in improving oral disease<sup>1)</sup> and preventing respiratory infection<sup>2-4)</sup>, and many studies have reported on the effects of periodontal disease<sup>5, 6)</sup> on the entire body.

A 2011 survey reported that pneumonia was the third most common cause of death in Japan

(<http://www.mhlw.go.jp/toukei/saikin/hw/jinkou/geppo/nengai11/kekka03.html>). Possible organisms causing pneumonia are *Streptococcus oralis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Escherichia coli*.<sup>7, 8)</sup> The vital statistics of Japan report that, in 2013, 24.1% of the population in Japan was >65 years of age and that 95% of patients with pneumonia were elderly

(<http://www.mhlw.go.jp/bunya/koyoukintou/josei-jitsujo/dl/12c-1.pdf>).

Pneumonia in the elderly has been suggested to be triggered by aspiration of bacteria residing in the oral cavity<sup>2-4</sup>), further highlighting the importance of oral care. It is thought that drinks may be an effective and easy way in which to administer agents to the elderly to prevent aspiration pneumonia. Although the antibacterial activity of coffee, black tea, and green tea against general bacteria, including *Serratia marcescens*, *Vibrio parahaemolyticus*, and *Staphylococcus aureus*, has been reported<sup>9-13</sup>), there have been fewer studies investigating antibacterial actions against oral bacteria.

In addition, the caffeine in these drinks has various side effects, including sleep disorders<sup>14-16</sup>). Thus, it is important to assess the antibacterial activities of drinks that do not contain caffeine.

Plants in the genus *Hypericum* (Guttiferae) have been used as medical herbs for a long time, and the antibacterial activities of *Hypericum perforatum* (Saint John's wort) have been reported<sup>17-22</sup>). Hypericin is one of the constituents of *H. perforatum* responsible for its antibacterial activity<sup>17</sup>); however, hypericin is known to cause some problems, including photosensitivity (hypericism<sup>19</sup>)).

In the present study, we examined the antibacterial activities of a hot water extract of *Hypericum erectum*, which is native to mountains in Japan, and confirmed, using HPLC analysis, that neither caffeine nor hypericin were present in the extract.

## **Materials and Methods**

### *Plant materials*

Four kinds of commercially available herbal tea were used: (i) leaves of *H. erectum* (Lot 51130606; Tochimoto Tenkaido, Osaka, Japan); (ii) fruits of *Crataegus cuneata* (product of Egypt; Lot BAD0205; Uchidawakanyaku, Tokyo, Japan); (iii) rose hip (i.e. fruits of *Rosa canina*; product of Chile; Seiwa no. 10296; All Life Service, Shizuoka, Japan); and (iv) German chamomile (i.e. flowers of *Matricaria rectita*; product of Egypt; Lot C1S3107; Uchidawakanyaku). The hot water extract of *H. erectum* (tea) was created by soaking 5 g *H. erectum* leaves with boiled distilled water (500 mL) for 5 min. To prepare the hot water extracts of *C. cuneata*, *R. canina*, and *M. rectita*, 5 g of the fruits or flowers was soaked in boiled distilled water (500 mL) for 5 min. All extracts were sterile filtered (0.45 µm pore

size; Millex-HV; Thermo Scientific, Waltham, MA, USA).

The isoquercitrin, quercitrin, quercetin, and rutin content in *H. erectum* tea is given in Table 1 (analyses performed by the Japan Food Research Laboratories, Tokyo, Japan).

### *Chemicals*

Caffeine was purchased from Nacalai Tesque (Lot M3H1447; Kyoto, Japan) and dissolved in distilled water at a concentration of 1 mg/ml. Hypericin was obtained from Sigma-Aldrich (Lot BCBJ9563V; St. Louis MO, USA) and dissolved in dimethyl sulfoxide (DMSO)(e.g. 100%) to a concentration of 1 mg/ml.

### *Bactericidal study*

The bacterial strains and the fungus used in the present study are listed in Table 2. Most bacterial strains were maintained anaerobically at 37°C in Brain Heart Infusion (BHI) broth (Difco Laboratories, Detroit, MI, USA). *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* were cultured in BHI broth containing 0.1% (v/v) hemin and 0.1% (v/v)

menadione. *Bacillus subtilis* and *Escherichia coli* were cultured aerobically at 37°C, whereas *Candida albicans* was maintained aerobically at 25°C in BHI broth. Overnight cultures of the microorganisms tested were harvested by centrifugation at 7000 r.p.m. for 5 min at 4°C and centrifugation speed is in 8,000 g. Cell pellets were washed three times with sterile phosphate-buffered saline (PBS) and adjusted to an optical density at 600 nm (OD<sub>600</sub>) of 0.5. The adjusted microorganism samples were diluted 1:1 000 000 in each tea sample. After 30 min exposure to the different teas, serial dilutions of the samples were plated onto BHI agar plates and incubated under appropriate conditions for 48 hr. The percentage of surviving microorganisms was calculated by counting the number of colony-forming units. Thus, percentage susceptibility was calculated as  $100 - (\text{the number of viable microorganisms after exposure to each tea sample}) / (\text{the number of viable cells after exposure to PBS}) \times 100$ .

### *Phytochemical analyses*

HPLC analyses for the presence of caffeine and hypericin were performed on Senshu Pak PEGASIL ODS SP100 (4.6Φ×150 mm; 1209063 H; Senshu

Scientific, Tokyo, Japan) and Mightysil RP-18 GP (4.6Φ×250 mm No. 25415-96; Kanto Chemical, Tokyo, Japan) columns, respectively. Wavelengths for caffeine and hypericin were monitored at 276 and 590 nm, respectively. The chromatographic method for both samples was isocratic. The eluent solvents used were H<sub>2</sub>O:methanol (6:4 v/v) for caffeine and 100 mM ammonium acetate:acetonitrile:methanol (20:30:50 v/v) for hypericin. The flow rate was set to 0.8 ml/min. Column temperature was adjusted to 40°C for caffeine and ambient temperature for hypericin.

A caffeine standard was created by dissolving caffeine in distilled water at a concentration of 1 mg/ml. Tea extracts were freeze dried to obtain powder and the powder was then dissolved in distilled water at a concentration of 1 mg/ml. A 20-μl aliquot of the sample was injected onto the column for HPLC analysis. For hypericin, a 1 mg/ml standard was created by dissolving hypericin in DMSO; the sample injection volume was 1 μl. As for caffeine analyses, *H. erectum* tea was freeze dried to obtain a powder. The powder was then dissolved in DMSO at a concentration of 10 mg/ml and a 5μl aliquot of this solution was injected onto the column for HPLC analysis (JASCO Corporation, Japan; HPLC conditions: PU-2089



Plus, MD-2010 Plus, CO-2065 Plus, LC-Net/ADC.).

Separation for rutin and quercetin was performed on a Unison UK-C18 column (4.6Φ×150 mm; Imtakt, Kyoto, Japan). For both samples, wavelength was monitored at 360 nm and a gradient chromatographic method was used. The eluent solvent used was 2.5% acetic acid:methanol (2:8 v/v) for both samples. The flow rate was set to 1.0 ml/min. Column temperature was adjusted to 40°C for both samples.

Separation for isoquercitrin and quercitrin was performed on Unison UK-C18 (4.6Φ×150 mm; Imtakt, Kyoto, Japan) and YMC-Pack ODS-AA12S05-15067WT (6.0Φ×150 mm; YMC, Kyoto, Japan) columns. Wavelength was monitored at 360 nm for both samples. Gradient and isocratic chromatographic methods were used for isoquercitrin and quercitrin, respectively. The eluent solvents used were methanol:acetonitrile:H<sub>2</sub>O:phosphoric acid for isoquercitrin and acetonitrile:2-propanol:H<sub>2</sub>O for quercitrin. The flow rate was set to 1.0 ml/min. The column temperature was adjusted to 50°C for isoquercitrin and to 40°C for quercitrin.

## Results

### *Bactericidal activity of tea extracts*

The broadest antibacterial activity was observed with the hot water extract of *H. erectum*. This extract had strong bactericidal effects against *Streptococcus gordonii*, *Streptococcus anginosus*, *Streptococcus salivarius*, *Streptococcus mutans*, *Streptococcus oralis*, *Streptococcus sanguinis*, *Streptococcus mitis*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *A. actinomycetemcomitans*, *P. gingivalis*, and *B. subtilis*. However, the hot water extract of *H. erectum* did not exhibit any antibacterial effects against *Actinomyces viscosus*, *Lactobacillus casei* var. *alactosus*, *Lactobacillus casei*, or *S. aureus*, or any effects against spores of *B. subtilis*, *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, or *C. albicans* (Table 2). The hot water extract of *C. cuneata* exhibited bactericidal effects against *S. oralis*, *S. pyogenes*, *A. actinomycetemcomitans*, *P. gingivalis*, and *B. subtilis*. However, the hot water extracts of *R. canina* and *M. rectita* only exhibited bactericidal effects against *S. oralis* and *P. gingivalis*.

### *HPLC analyses*

HPLC analysis revealed the presence of isoquercitrin, quercitrin, and quercetin in the *H. erectum* tea, but rutin levels were below the limit of detection (Table 1).

HPLC analysis of the caffeine standard revealed a clear peak at a retention time (Rt) of 3.24 min. In the vehicle (distilled water) sample, there was a peak at 1.95 min. There were no peaks at the caffeine Rt for samples of the hot water extracts of *H. erectum*, *C. cuneata*, *R. canina*, or *M. rectita* (Fig. 1).

HPLC analysis of the hypericin standard revealed a clear peak at a Rt of 23.36 min, whereas in the vehicle (DMSO) standard there was a peak at Rt 3.89 min. There were no peaks corresponding to hypericin in the *H. erectum* sample (Fig. 2), indicating that this extract contains no hypericin or that the hypericin content in this extract is below the limit of detection.

## **Discussion**

Aspiration of bacteria residing in the oral cavity has been implicated as a cause of pneumonia in older people<sup>2, 7, 8</sup>). It is now recognized that appropriate oral care can prevent aspiration pneumonia<sup>2,7</sup>). The consumption of drinks may provide an effective and easy approach to good oral care in the

elderly. Coffee, black tea, green tea, oolong tea, and *H. perforatum*, an ancient herbal remedy, are popular and widely consumed beverages worldwide. However, the caffeine in these drinks has been reported to cause sleep disorders, as well as affecting fetal development<sup>12-14</sup>). Hypericin, one of the major photosensitizing constituents of *H. perforatum*, is considered to be responsible for hypericium<sup>17</sup>). In the present study, we selected four teas that are believed to be caffeine free (i.e. *C. cuneata*, *R. canina*, *M. rectita*, and *H. erectum*) and investigated whether these teas contain caffeine using HPLC analysis. To evaluate the actions of the teas when they are consumed as usual, extracts were prepared according to the suppliers' instructions. HPLC analysis revealed that the extracts of the four teas (i.e. *H. erectum*, *C. cuneata*, *R. canina*, and *M. rectita*) did not contain the peak seen with the caffeine standard (Fig. 1). These results suggest that four herbal teas tested do not contain caffeine. Because *H. erectum* belongs to the genus *Hypericum*, it was possible that the hot water extract of *H. erectum* could contain hypericin. However, based on results of HPLC analysis, hypericin was not present in the hot water extract of *H. erectum*, or, if it was, it was present at levels below the limit of detection (Fig. 2). The antibacterial activities of coffee,

black tea, green tea, and *H. perforatum* against various bacteria, as well as effects against fungi, have been reported previously<sup>9-11)</sup>. Coffee extracts have been suggested to have inhibitory actions against *Proteus hauseri* and *Serratia marcescens*<sup>9)</sup>. Daglia *et al.* reported that components from roasted coffee exhibit bactericidal activity against *S. aureus*, but not *S. mutans*<sup>10)</sup>. Tea extracts have been suggested to be useful as prophylactic agents against *Mycoplasma pneumoniae* infection<sup>12)</sup>. It has been reported that extracts of Japanese green tea leaves inhibit the growth of various bacteria causing diarrheal diseases<sup>13)</sup>. Despite these reports of the effects of common drinks against various bacteria, there is little information regarding the effects of drinks against oral bacteria. To date, the effects of coffee, green tea, and oolong tea against *S. mutans* have only been reported<sup>10, 11)</sup>. Thus, the present study primarily evaluated the antibacterial effects of the teas against oral bacteria, such as oral streptococci. As indicated in Table 2, except for the effect of *C. cuneata* against *A. actinomycetemcomitans*, the strongest and broadest antibacterial effects were seen for the *H. erectum* extract. In the present study, we did not detect any bactericidal effects of the *H. erectum* extract against *E. coli* or *P. aeruginosa*, or any fungicidal effects against *C.*

*albicans*. These findings are in contrast with those reported for *H. perforatum*, another *Hypericum* species, which has been shown to exhibit antibacterial activity against Gram-positive bacteria (*S. aureus* and *E. faecalis*) and Gram-negative bacteria (*E. coli* and *P. aeruginosa*)<sup>17)</sup>. The apparent discrepancy may be due to differences in the constituents extracted by hot water. One of the important components of *Hypericum* species responsible for antibacterial activity is hypericin<sup>17)</sup>. Hypericin may play an important role in killing *E. coli*, *P. aeruginosa*, and *C. albicans*. As shown in Fig. 2, hypericin was not detected in the hot water extract of *H. erectum*, and this could explain why this extract had no effect against *B. subtilis*, *E. coli*, *K. pneumoniae*, *P. aeruginosa* or *C. albicans* in the present study. Together, these results suggest that the hot water extract of *H. erectum* effectively kills oral bacteria, especially oral streptococci. It has been reported<sup>23)</sup> that the bactericidal effect following gargling is maintained for 30 min. Because 30 min exposure to tea extract is sufficient to kill oral streptococci, *H. erectum* could be used as an oral gargling agent to prevent aspiration pneumonia based on its antibacterial effects in the present study against *S. oralis* and *S. aureus*, which are known to cause aspiration pneumonia. Evaluation of the

bactericidal effects of the *H. erectum* extract demonstrated that it is effective not only against oral streptococci, but also against periodontal pathogenic bacteria, such as *P. gingivalis* and *A. actinomycetemcomitans*. Therefore, we hypothesize that drinking *H. erectum* may be useful not only for the prevention of aspiration pneumonia in the elderly, but also for the prevention of caries and periodontal disease. The *H. erectum* extract was found to contain quercetin and quercitrin<sup>24)</sup>, and it has been reported that the antimicrobial activity of quercetin and quercitrin increases synergistically compared with either compound alone<sup>25)</sup>. Because both quercetin and quercitrin were found to be components of the *H. erectum* tea in the present study, the antibacterial effect of this extract may have been due to a synergistic action between the two.

Coumarin, xanthone, and piperidine, which have been shown to have antibacterial actions, are present in an organic solvent extract of Guttiferae<sup>26, 27)</sup>. In the present study we examined the antibacterial effects of hot water extracts, as could be used clinically for oral care (e.g. *H. erectum* tea). The concentrations of antibacterial compounds are known to vary depending on the part of the plant used and the method of extraction<sup>28)</sup>. The antibacterial

compounds extracted using organic solvents may also be present in hot water extracts. Therefore, in future studies we will examine levels of organic solvent-extracted antibacterial compounds, including coumarin, in hot water extracts.

Even though the *H. erectum* extract was found to exert significant effects against oral bacteria, the constituents responsible for this bactericidal activity have not been identified. Taking into consideration the potential side effects of drinks, further detailed analysis to identify the constituents of the hot water extract of *H. erectum* must be undertaken. We believe that the hot water extract of *H. erectum* may be an easy, safe, and effective agent to prevent aspiration pneumonia in the elderly.

### **Conflict of interest**

The authors declare they have no conflicts of interest.

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## Figure Legends

Fig. 1. HPLC chromatograms of the caffeine standard and hot water extracts from *Hypericum erectum*, *Crataegus cuneata*, *Rosa canina*, and *Matricaria rectita*. Each extract was prepared according to the suppliers' instructions. The peak for caffeine, and its retention time, is indicated by the arrow.

( Minimum detection area was 0.27 mg/ml )

Fig. 2. HPLC chromatograms of the hypericin standard and the hot water extract of *Hypericum erectum*. The peak for hypericin, and its retention time, is indicated by the arrow.

( Minimum detection area was 2.25 mg/ml )

**Table 1. Phytochemical analyses, performed by Japan Food Research Laboratories**

Compound	Weight ( <i>H. erectum</i> leaves)	Minimum detection area	Analytical method
Isoquercitrin	4.6 mg/100 g		HPLC
Quercitrin	7.3 mg/100 g		HPLC
Quercetin	16 mg/100 g		HPLC
Rutin	<u>Not</u> detected	0.5 mg/100 g	HPLC



**Table 2. Susceptibility of different microorganisms to the four herbs tested (% susceptibility)**

<b>Species</b>	<b><i>H. erectum</i></b>	<b><i>C. cuneata</i></b>	<b><i>R. canina</i></b>	<b><i>M. rectita</i></b>
<i>S. gordonii</i> ATCC 10558	100	47	0	0
<i>S. anginosus</i> IS 57	100	0	0	0
<i>S. salivarius</i> KT 12	100	88	78	46
<i>S. mutans</i> 109 C	100	49	0	0
<i>S. oralis</i> ATCC 35037	100	99	99	98
<i>S. sanguinis</i> ATCC 10556	100	49	49	49
<i>S. mitis</i> ATCC 49456	100	49	0	0
<i>S. pyogenes</i> ATCC 12348	100	98	0	0
<i>S. pneumoniae</i> IID 553	78	49	49	50
<i>A. viscosus</i> ATCC 15987	7	0	0	0
<i>A. actinomycetemcomitans</i> Y 4	95	100	95	0
<i>L. alactosus</i> JCM 1133	0	0	0	0
<i>L. casei</i> JCM 1134	48	0	0	0
<i>P. gingivalis</i> ATCC 3327	100	100	100	100
<i>S. aureus</i> ATCC 6538	76	0	0	0
<i>B. subtilis</i> ATCC 9372	100	98	44	0
<i>B. subtilis</i> ATCC 9373 (spore)	0	0	0	0
<i>E. coli</i> K 12	0	0	0	0
<i>K. pneumoniae</i> IID 5209	0	0	0	0
<i>P. aeruginosa</i> ATCC 27853	0	0	0	0
<i>C. albicans</i> JCM 2085	0	0	0	0

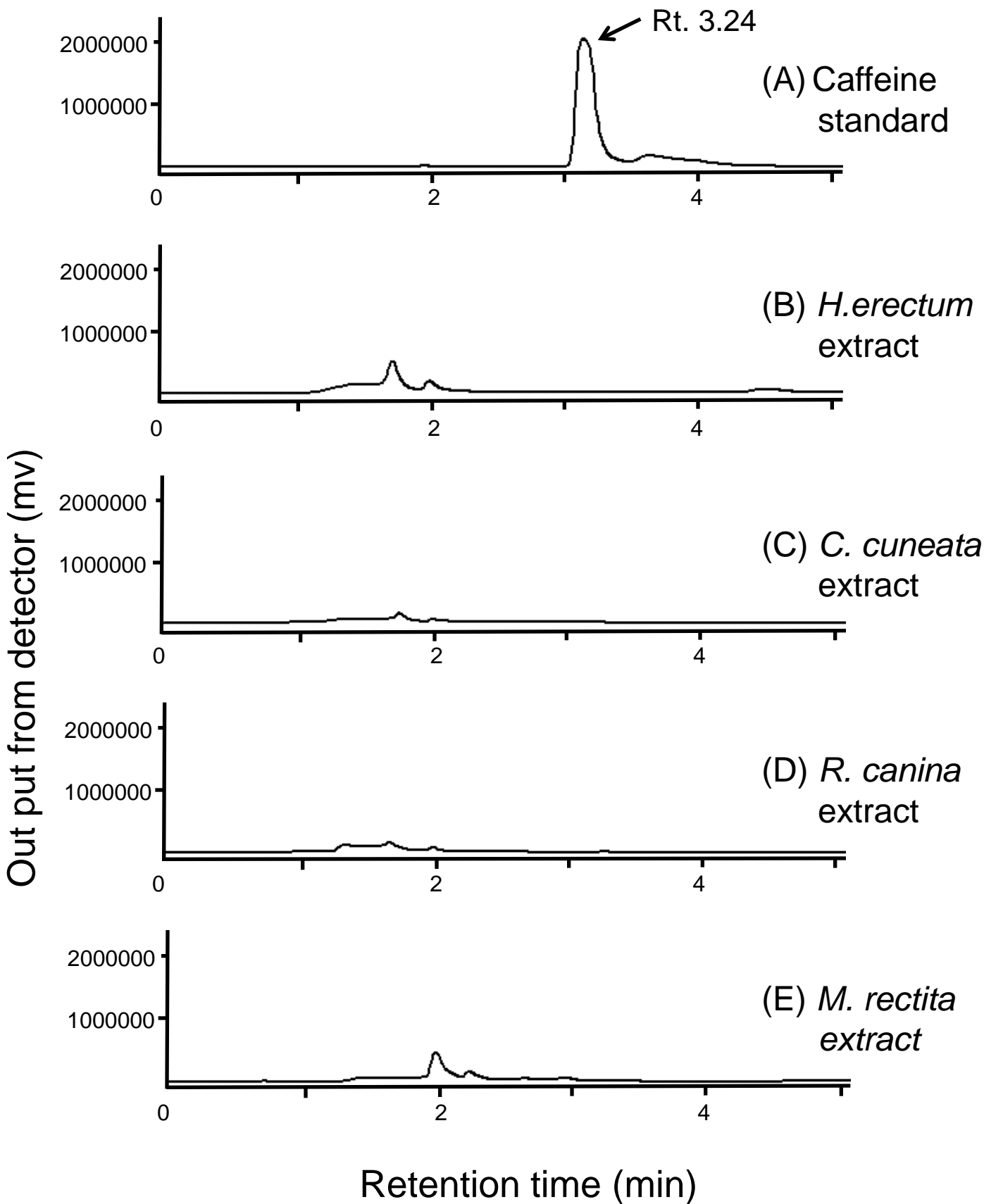


Figure 1

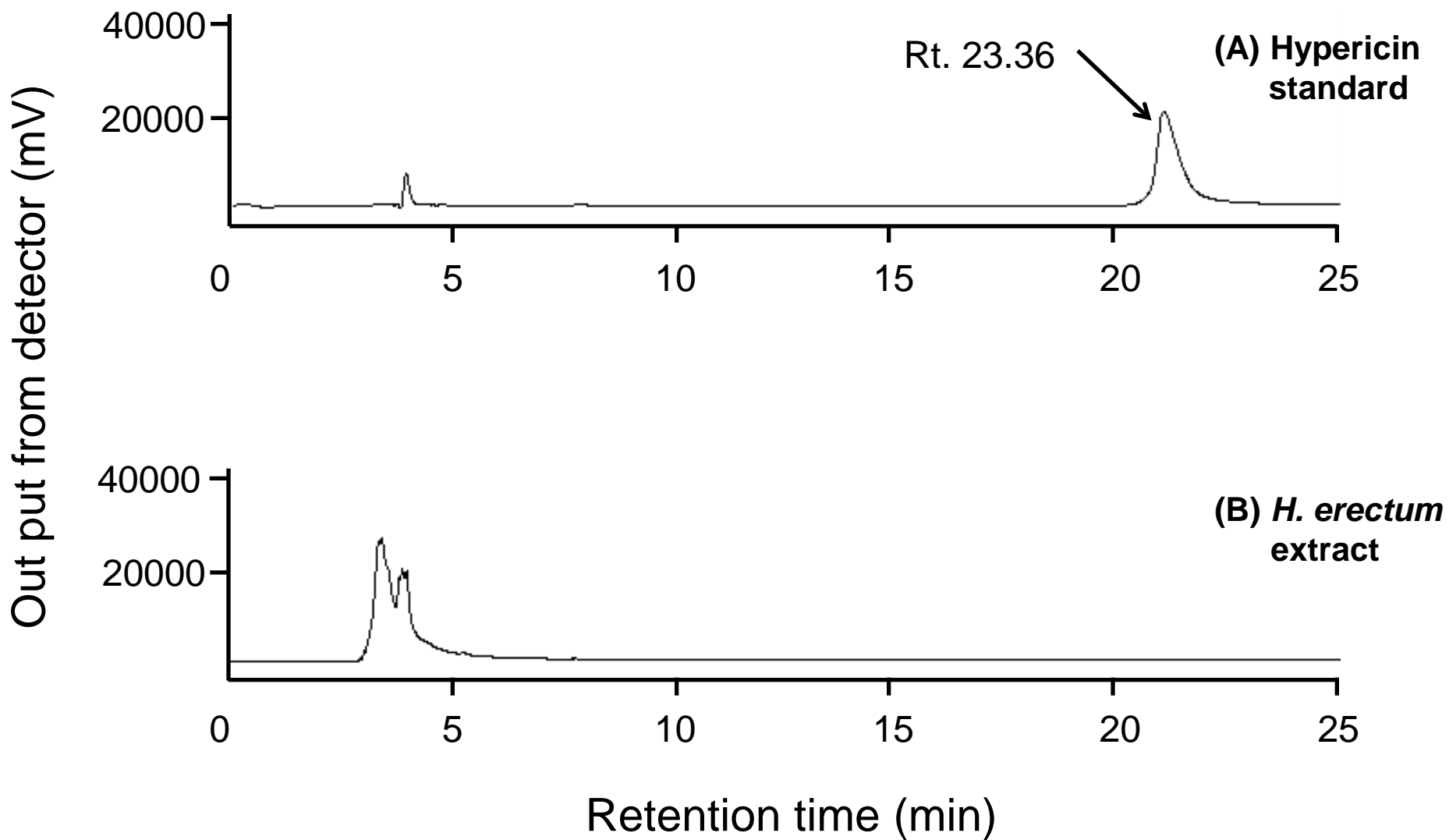


Figure 2