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Benzoquinone, the substance essential for antibacterial activity in aqueous extracts from succulent young shoots of the pear *Pyrus* spp.

Shigeki Jin*, Norio Sato

National Agricultural Research Center for Hokkaido Region, Toyohira-ku, Sapporo 062-8555, Japan

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Abstract

Aqueous extracts of the tissue of succulent young shoots of the pear *Pyrus* spp. exhibited strong antibacterial activity against the bacterium *Erwinia amylovora* bv. 4. This activity was investigated quantitatively by a newly developed bioassay method. It was found that the activity changed with the age of the tissue. Extracts of the youngest leaves and stems from the shoot tops showed the strongest activity, and the activity decreased with age of the leaves and stems. The activity also changed with increase in time after preparation of the extract, increasing rapidly in the first hour after preparation, reaching a maximum at about 4 h, and then decreasing slowly. The substance essential for the antibacterial activity was isolated from the extract by steam distillation in vacuo and through charcoal powder column chromatography. It was identified as benzoquinone (2,5-cyclohexadiene-1,4-dione) by NMR-spectra, mass spectra and HPLC analysis. The phenolic metabolism from arbutin to hydroquinone and then to benzoquinone in the aqueous extracts was analyzed quantitatively by HPLC. The changes in the contents of benzoquinone in the extracts of leaves and stems with tissue aging and with increase in time after preparation of the extracts paralleled the changes in antibacterial activity as determined by the quantitative bioassay.

Keywords: Pear; *Pyrus ussuriensis* Maxim.; Rosaceae (III); *Erwinia amylovora*; Antibacterial activity; Benzoquinone; Arbutin; Hydroquinone; Bacterial shoot blight disease

1. Introduction

Plant defense reactions against infection and growth of pathogens in plant tissue are generally accompanied by localized browning of affected tissues, and the browning phenomenon appears to play an important role in the inhibition of proliferation of pathogens. Browning is known to occur due to the polymerization and chemical changes in quinones produced through phenol metabolism. Therefore, antimicrobial activities of phenolic compounds and their metabolism was one of the most important research fields in phytopathological chemistry many years ago. However, free phenolic compounds and their glucosides generally exhibited low activities, and the important quinones had not been analyzed presumably because of the preconception that quinones are too unstable to exist in free form in plants (Farkas and Király, 1962; Kosuge, 1969).

Stems and leaves of succulent young shoots of the pear (*Pyrus* spp.) exude some substances with strong antibacterial activity when the tissues are injured. Aqueous extracts of the tissues also exhibit strong antibacterial activity. Hildebrand and Schroth (1963, 1964a,b) tried to elucidate the antibacterial activity in pear using a semiquantitative bioassay, the so-called inhibition zone method, with *Erwinia amylovora* (Burr.) Winslow et al., which is the causal agent of fire blight disease in pears. Their results clearly showed that β-glucosidase was the key enzyme in the production of the active substance. Although β-glucosidase metabolized arbutin **1** to hydroquinone **2**, hydroquinone **2** was seldom detected in the aqueous extract and exhibited only low activity. The active substance disappeared during isolation, and Hildebrand and Schroth could only speculate that an unstable semiquinone might be the essential substance. On the other hand, Smale and Keil (1970) detected abundant hydroquinone **2** in pear leaves, and they suggested that hydroquinone **2** is an important substance. However, in a later study, the

* Corresponding author. Fax: +81-11-859-2178.
E-mail address: sjin@naro.affrc.go.jp (S. Jin).

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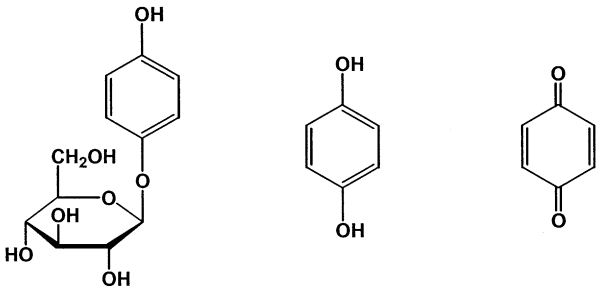

1 2 3

1: arbutin
2: hydroquinone
3: benzoquinone

abundance of hydroquinone **2** was found to be an artifact in preliminary treatments (Hildebrand, 1970). Following this revelation, research on the antibacterial substance present in pear was apparently discontinued.

In an attempt to identify the antibacterial substance in pears, we used a Chinese pear (*Pyrus ussuriensis* Maxim.) was used to prepare aqueous extracts and *E. amylovora* bv. 4 as a test bacterium. A new quantitative bioassay method was then developed and a careful search for the antibacterial substance was conducted. The active substance was isolated, identified, and analyzed quantitatively, and the results are reported here.

2. Results

2.1. Relation of tissue aging to antibacterial activity

Three stem pieces, top (5 cm from the tip), second (5–10 cm from the tip) and third (10–15 cm from the tip) parts, were sampled from a shoot, and respective aqueous extracts were tested for antibacterial activity. Each extract was prepared in 20 ml of aqueous solution regardless of fresh sample weight. The time from disruption of tissues to antibacterial assay was 1 min, and the time from addition of the test bacterium to the aqueous extract until inoculation on the selective agar medium was also 1 min. Five leaves from a shoot, from the top folded soft one to the fifth open and comparatively hardened one, were also similarly tested. The results are shown in Table 1. The aqueous extract from the youngest part of the stem showed the strongest antibacterial activity; that is, colony formation was completely inhibited. With increase in age of each part of the stem, the increasingly hardened tissues gave extracts with decreasing activity. The results of tests on leaves were similar to those of tests on stem pieces.

Table 1
Antibacterial activities of aqueous extracts from different parts of a young stem and a leaf of a pear (each value is the mean ± SD)

Part	Position	% Inhibition	(Individual data)
Stem	Top (5 cm from tip)	100	(100, 100, 100, 100, 100)
	2nd (5–10 cm from tip)	85 ± 12	(100, 99, 95, 93, 37)
	3rd (10–15 cm from tip)	57 ± 11	(95, 66, 45, 41, 37)
Leaf	Top	100	(100, 100, 100, 100)
	2nd	99 ± 0.5	(100, 99, 98, 98)
	3rd	98 ± 1	(100, 99, 98, 95)
	4th	94 ± 2	(99, 93, 91, 91)
	5th	87 ± 4	(97, 89, 84, 79)

2.2. Relation of time after extract preparation to antibacterial activity

When the shoot top tissues were macerated, the aqueous extracts changed from nearly colorless to a yellow-orange color and the color became increasingly denser with time, indicative of some rapid chemical changes. Therefore, the relation between preparation time and antibacterial activity was investigated. An aqueous extract (100 ml) was prepared from two pieces of top stem (0.5 g) and stirred for 24 h. At prescribed times (10, 20 and 40 min and 1, 2, 4, 6, 8, 12 and 24 h after preparation of the extract), 0.1 ml of the extract was withdrawn, diluted with 0.8 ml of phosphate buffer, and mixed with 0.1 ml of bacterial suspension, giving a test solution of 2000-fold dilution of fresh weight. Two minutes later, the test solution was inoculated on a selective agar medium. The results are shown in Fig. 1. Antibacterial activity increased rapidly within 1 h after preparation of the extract, reached maximum at around 4 h, and then decreased slowly. The same experiments were repeated several times, and similar results were obtained.

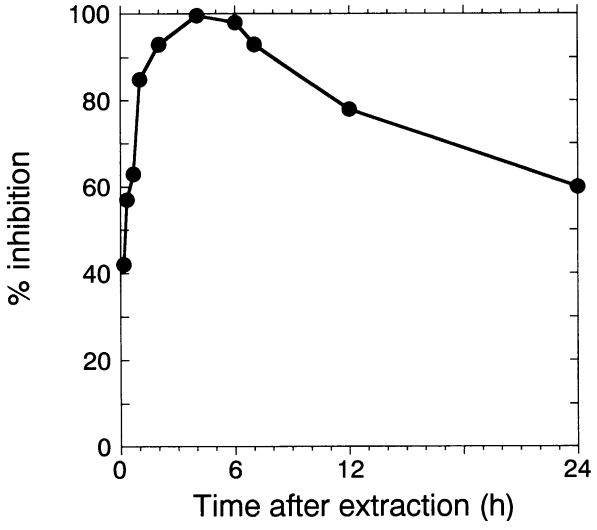

Fig. 1. Changes in antibacterial activity of an aqueous extract of a young shoot.

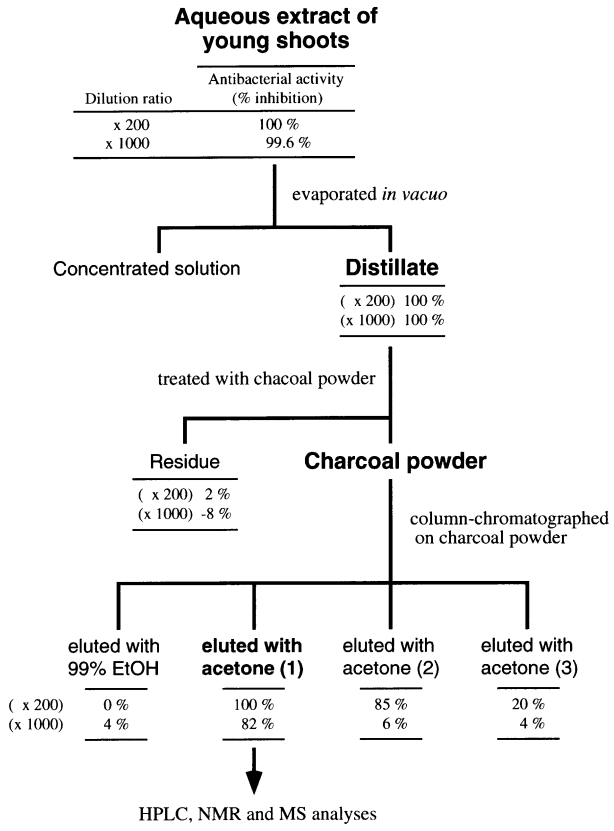

Fig. 2. Scheme for purification of the essential active substance together with bioassay data.

2.3. Isolation and identification of the essential active substance

The results of the above-described experiments clearly indicated that aqueous extracts of young shoot tissues produced some strong antibacterial substances. Therefore, the essential substance was searched for using a quantitative bioassay. The isolation scheme is shown in Fig. 2 together with the bioassay data. An aqueous extract (200 ml) of young stems and leaves (10 g) was stirred for 2 h to increase antibacterial activity. The yellow-orange extract was evaporated in vacuo, and the whole activity was recovered in the yellow distillate. The distillate, after being mixed with charcoal powder, lost its yellow color and antibacterial activity at the same time. A yellow substance was eluted by acetone from a charcoal powder column. The dense yellow acetone eluate exhibited strong antibacterial activity. A single substance was found in this acetone eluate and identified as benzoquinone **3** by HPLC, NMR and MS analyses.

2.4. Changes in contents of benzoquinone *3* and related substances with time after preparation of the extract

As stated above, the antibacterial activity of the aqueous extract changed with preparation time. If benzoquinone **3** is the essential substance, its content in the aqueous extract should also change with time. Therefore, the relation between preparation time and content of benzoquinone **3** was investigated. Four stem pieces, top (5 cm from the tip), second (5–10 cm from the tip), third (10–15 cm from the tip), and fourth (15–20 cm from the tip) parts, were sampled from shoots, and respective aqueous extracts were analyzed at prescribed times (1, 3, 6, 9, 12 and 24 h after preparation of the extract). A sample at 0 h was prepared by homogenizing the stem piece in 99% EtOH. The results are shown in Fig. 3. Benzoquinone **3** and hydroquinone **2** were not detected in any parts of stem at 0 h, but arbutin **1** was detected. The amount of benzoquinone **3** in the top part increased rapidly within 1 h after preparation of the extract, reached a maximum value at around 9 h, and then decreased slowly. The amount of arbutin **1** decreased in inverse proportion to that of benzoquinone **3**. The amount and the rate of increase in the amount of benzoquinone **3** decreased with increase in age of the stem part. Leaves in three zones in which young shoots were attached, first (5–15 cm from the tip), second (15–25 cm from the tip), and third (25–35 cm from the tip) zones, were also analyzed similarly. The results, which are shown in Fig. 4, were similar to those of stem analysis.

2.5. Antibacterial activities of benzoquinone *3* and hydroquinone *2*

The antibacterial activities of phosphate buffer solutions of benzoquinone **3** and hydroquinone **2** were tested, and the results are shown in Table 2. In a solution of 5 ppm benzoquinone **3**, colony formation was completely inhibited during treatment with the test bacterium for 2 min. Moreover, in the case of treatment for 10 min, solutions of 1–2 ppm benzoquinone **3** inhibited colony formation completely. On the other hand, hydroquinone **1** did not completely inhibit colony formation even with a solution of 2000 ppm during a 10-min period of treatment with the test bacterium.

3. Discussion

It is well known that an extract of succulent young shoot tissue of pear exhibits strong antibacterial activity. This activity was also found in extracts of 12 cultivars of Chinese pears (*P. ussuriensis*), Japanese pears (*P. pyrifolia*) and European pears (*P. communis*) in our preliminary experiments (data not shown). In our present experiments using a quantitative bioassay, even extracts of 1000–2000-fold dilution per fresh weight of tissue were able to kill the mixed test bacterium within 2 min. In this study, we succeeded for the first time in isolating the active substance that is essential for the antibacterial activity. The substance was identified as

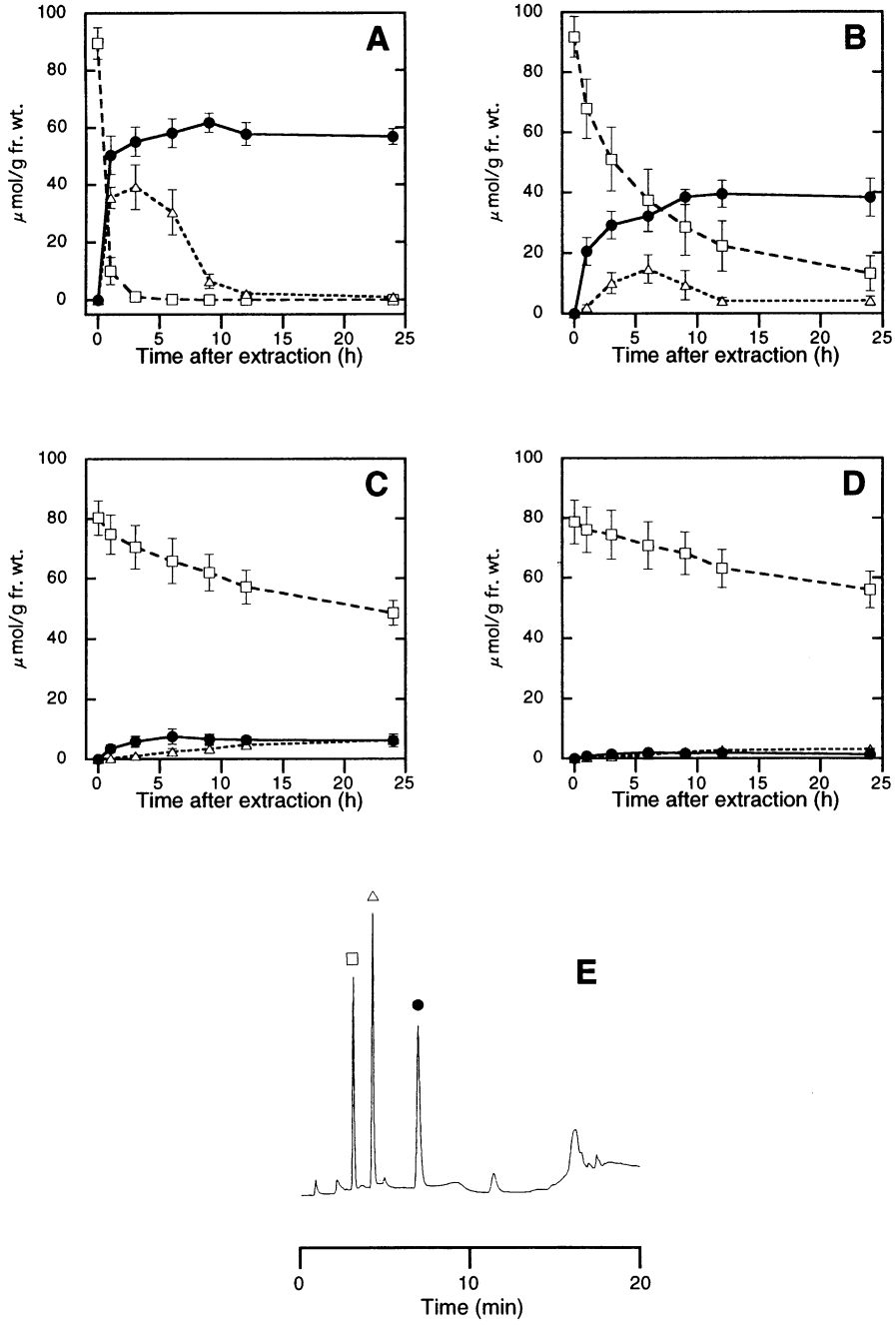

Fig. 3. Changes in amounts of compounds in aqueous extracts of the top part (A), second part (B), third part (C) and fourth part (D) of a stem of a pear with increase in time after extraction, and the HPLC chromatogram of an aqueous extract at UV 220 nm (E). Solid circles, benzoquinone **3**; open squares, arbutin **1**; open triangles, hydroquinone **2**.

benzoquinone **3**, and its amounts were analyzed chemically. The content of benzoquinone **3** in the aqueous extracts changed with time, and the changes paralleled the changes in antibacterial activity as determined by the bioassay, again indicating that the essential substance is benzoquinone **3**. The phenol metabolism from arbutin **1** to hydroquinone **2** and then to benzoquinone **3** was also elucidated quantitatively. Arbutin **1** is an inactive substance and is thought to be a stock substance for the defense reaction. Hydroquinone **2**, which has only slight activity, did not accumulate in a sufficient amount to exhibit antibacterial activity and is thought to be an intermediate substance. Benzoquinone **3**, the oxidized product of hydroquinone **2**, not only exhibited remarkably strong activity but also accumulated in large amounts, and is therefore thought to be the substance essential for the defense reaction.

Why has benzoquinone **3**, which not only has strong antibacterial activity but is also capable of abundant accumulation, not been considered as a candidate for

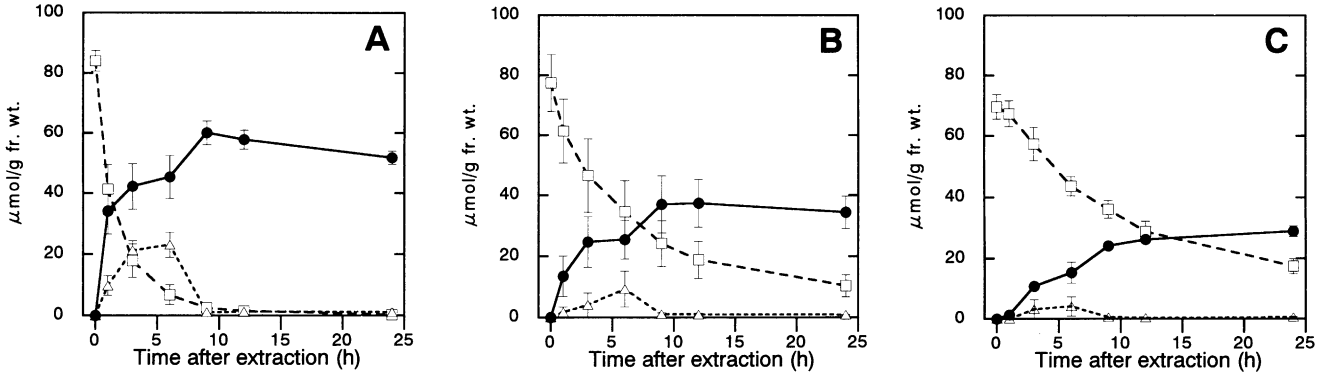

Fig. 4. Changes in amounts of compounds in aqueous extracts of leaves in the first zone (A), second zone (B) and third zone (C) with increase in time after extraction. Solid circles, benzoquinone **3**; open squares, arbutin **1**; open triangles, hydroquinone **2**.

Table 2
Antibacterial activities of benzoquinone **3** and hydroquinone **2**

Treatment time (min)	% Inhibition				
	Concentration of benzoquinone **3** (ppm)				Concentration of hydroquinone **2** (ppm)
	5	2	1	0.5	2000
2	100	94	65	32	12
10	100	100	99	89	31

the substance essential for the antibacterial activity? One reason may be the inadequate bioassay, i.e. the so-called inhibition zone method used in previous studies. This method does not enable analysis of momentary change in activity. Our new quantitative bioassay, on the other hand, enabled monitoring of changes in the activity of the antibacterial substance easily. An excellent selective medium, which is essential for this bioassay, was developed by one of the authors (Sato). This selective medium (named "TSCC") completely inhibited activities of bacteria such as *Erwinia herbicola* and *Pseudomonas fluorescens*, which are habitants of the surfaces of pear shoots, but facilitated colony formation of *E. amylovora* bv. 4. This quantitative bioassay method should also be useful to search for antibacterial substances in other plants.

Another possible reason for benzoquinone **3** not having been considered as the essential substance is the preconception that quinones are too unstable to exist as free forms not only in plants but also in extract solutions (Farkas and Király, 1962; Kosuge, 1969). However, our results indicated that benzoquinone **3**, at least, was stable not only for the bioassay but also for chemical analysis of the extract solutions. One more possible reason for benzoquinone **3** not having been considered as the essential substance is its physical nature; i.e. steam distillation in vacuo. Braghiroli et al. (1996) recently carried out experiments to try to identify the antibacterial substance in *Ericaceae* plants from aqueous extracts of plant tissues, but they lost the active substance during the processes of isolation and purification. Finally, they identified arbutin **1** and hydroquinone **2**, but their antimicrobial activities were extremely weak compared to those in the original aqueous extracts. Evaporation in vacuo was used to concentrate solutions at some steps of the isolation procedure. From our experimental experience, it can be presumed that the essential active substance was benzoquinone and that it was lost during the evaporation process.

Thus, the antibacterial substance in the aqueous extracts of pear shoot tissues was finally identified by a careful search using a newly developed bioassay method in the present study.

4. Experimental

4.1. Plant material and preparation of aqueous extracts for antibacterial assay

Succulent young shoots of a Chinese pear (*P. ussuriensis* Maxim., cv. Mishirazu) were sampled from pear trees in the field of the National Agricultural Research Center for Hokkaido Region, Sapporo, Japan. The shoots were separated into stems and leaves, and each part of the tissues was macerated in a mortar or homogenized in phosphate buffer (10 mM, pH 6.0) using a homogenizer. After extraction, the suspensions were filtered through gauze to remove plant debris.

4.2. Bacterium used for tests of antibacterial activity

The isolate YPPS-117 of *E. amylovora* bv. 4 (Mizuno et al., 2000), the causal agent of bacterial shoot blight of pears in Japan, kindly donated by Yokohama Plant Protection Station, was used as the test bacterium in this study. The bacterium was grown for 2 days at 25 °C on agar slants of a medium of the following composition: 1 l distilled and deionized water, 24 g nutrient agar

(DIFCO), 5 g yeast extract (DIFCO), 10 g sucrose, 10 g D-sorbitol, 2 g KH_2PO_4, and 4 g $Na_2HPO_4 \cdot 12H_2O$. Bacterial suspensions of two concentrations (A, $2–4\times10^5$ cfu/ml; B, $1–2\times10^4$ cfu/ml) were prepared by dilution with 0.1% NaCl solution.

4.3. Quantitative bioassay for antibacterial activity

In experiments to determine relation of tissue aging to antibacterial activity, the whole aqueous extract (20 ml) of a sample tissue was mixed with bacterial suspension A (0.1 ml) to give a test solution for antibacterial activity. After 1 min of mixing, the test solution (0.1 ml) was spread on an agar plate in a 9 cm diameter petri dish containing the selective medium described below.

In experiments for determining relation of time after preparation of the extract to antibacterial activity, an aliquot (0.9 ml) of aqueous extract (100 ml) was mixed with bacterial suspension B (0.1 ml) to give a test solution. After 2 min of mixing, the test solution (0.1 ml) was spread on an agar plate.

In both experiments, phosphate buffer mixed with bacterial suspension was used as a control test solution. In most cases, two plates were inoculated for each test solution. The inoculated petri dishes were sealed with parafilm tape to retain air moisture in the dishes, and the dishes were kept at 25 °C for 2–3 days to allow bacterial colonies to form. No contaminating bacterial colonies were detected. Only the test bacterium *E. amylovora* grew well and formed distinct colonies. In almost all cases, the number of colonies in a control petri dish ranged from 100 to 200. The numbers of colonies in the petri dishes for each test solution were counted, and the relative number (N) to the control (100) was calculated. The antibacterial activity is expressed as the percent of inhibition of colony formation [$100-N$].

4.4. Selective medium (TSCC)

The composition of the selective medium and its preparation procedure are as follows. Nutrient agar (12 g, DIFCO), D-sorbitol (20 g), KH_2PO_4 (1 g), $Na_2HPO_4 \cdot 12H_2O$ (2 g), sodium dodecyl sulphate solution (1 ml, 50 mg/ml EtOH), crystal violet solution (1 ml, 2.5 mg/ml EtOH), and bromthymol blue solution (1 ml, 20 mg/ml 0.1 N aq. NaOH) were added to of distilled deionized water (500 ml) in a bottle, which was then heated until boiling began, this being continued for 40 min in order to dissolve agar. Then, while stirring the resulting solution, cycloheximide solution (1 ml, 50 mg/ml EtOH) and thallium nitrate solution ($TlNO_3$, 1.5 ml, 50 mg/ml H_2O) were added, and the bottle was placed in a water bath (56–60 °C) for 30 min. After stirring for a few minutes, the medium was transferred into 9 cm diameter petri dishes. After the agar had solidified, each dish was sealed with parafilm tape to prevent drying of the agar plate, and the dishes were kept at room temp in a dark cabinet. Under these conditions, the agar plates were stable for at least 3 months. This medium was given the name TSCC from the initials of thallium (nitrate), sodium dodecyl sulphate, crystal violet, and cycloheximide, respectively.

4.5. Isolation and identification of antibacterial substance

Stems and leaves (10 g, total) were cut from shoot tops and blended in 10 mM phosphate buffer pH 6.0 (200 ml). After extraction, the suspension was filtered through a sheet of gauze and stirred (2 h, room temp). The extract was centrifuged (3000 rpm, 10 min) to remove the residue. The clear extract was evaporated in vacuo at 30 °C bath temp and 1 °C coolant temperature. The distillate solution (190 ml), which has a yellow color, was mixed with charcoal powder (1 g, Norit A; Norit Co.) and shaken on a shaker until the solution was colorless. The charcoal powder was collected by centrifugation and suspended in 90% EtOH (10 ml). The suspension was poured over a preliminarily prepared charcoal column (1 g), giving a total of 2 g of charcoal in the column. The charcoal column was washed with 90% EtOH (10 ml) and eluted with 99% aq. EtOH (20 ml) and then acetone (3×20 ml). The first acetone fraction, which was colored deep yellow, exhibited the strongest antibacterial activity. This fraction was analyzed by HPLC, NMR and MS and found to contain a single substance, which was identified as benzoquinone **3**. The NMR spectral data and MS data are as follows: ^{1}H NMR (300 MHz, $CDCl_3$) β: 6.79 (4H, *s*, H-2, 3, 5, 6). ^{13}C NMR (75 MHz, $CDCl_3$) β 187.0 (C-1, 4), 136.5 (C-2, 3, 5, 6). Negative MS (ESI): m/z 108 $[M]^-$. The spectral data and results of HPLC analysis of an authentic sample correspond exactly with those of the isolated natural product.

4.6. Quantitative HPLC analysis

Sample solutions for quantitative analysis of benzoquinone **3** and related compounds were prepared by homogenizing tissue (0.5 g) in H_2O (20 ml). HPLC analyses were performed using an ODS column (Wakosil-II 5C18HG, 150×4.6 mm in i.d.; Wako Pure Chemical Industries, Ltd.) with UV detection at 220 nm. The column was eluted at 30 °C at a flow rate of 0.8 ml/min with 15% aq. MeOH. After filtration of a sample solution with a membrane filter (0.45 μm, mixed cellulose ester; Advantec), an aliquot (10 μl) of the sample solution was injected. Under these conditions, arbutin **1**, hydroquinone **2** and benzoquinone **3** were eluted at 3.1, 4.2 and 7.0 min, respectively.

4.7. Apparatus

The ^{1}H and ^{13}C NMR spectra were measured at 300 and 75 MHz with an AMX-300 instrument (Bruker). The MS data were obtained by electrospray under atmospheric pressure, using direct injection (6 μl min^{-1}) of a solution of MeOH–H_2O–HOAc (50:50:0.1), with an API-100 instrument (Perkin-Elmer SCIEX; ionspray voltage of −4.0 kV [negative mode]; orifice voltage of −40 V; nebulizer gas, air; curtain gas, nitrogen).

4.8. Chemicals

Authentic samples of chemicals, arbutin, hydroquinone and benzoquinone, were purchased from Wako Pure Chemicals.

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