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RESEARCH ARTICLE

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Comparative Analysis of Anticancer and Antibacterial Activities among Seven *Trametes* Species

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ABSTRACT

Species in the genus *Trametes* (*Basidiomycota, Polyporales*) have been used in natural medicine for a long time. Many studies reported that mycelia or fruiting bodies of *Trametes* spp. exhibited effects of antioxidant, anti-inflammatory, anticancer, and antimicrobial activities. However, comparative analysis in this genus is scarce due to limitation of morphological identification and the sample number. In this study, the 19 strains of seven *Trametes* species were chosen to generate a five-gene-based phylogeny with the 31 global references. In addition, 39 culture extracts were prepared for 13 strains to test for anticancer and antibacterial activities. Strong anticancer activities were found in several extracts from *T. hirsuta* and *T. suaveolens*. Anticancer activities of *T. suaveolens*, *T.* cf. *junipericola* and *T. trogii* were first described here. The antibacterial ability of *T. versicolor* and *T. hirsuta* extracts has been confirmed. The antibacterial activities of *T. suaveolens* have been reported at the first time in this study. These results suggest an efficient application of the genus *Trametes* as the drug resources especially for anticancer agents.

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1. Introduction

Mushrooms are valuable for their content of various nutrients, including organic acids and polysaccharides as well as for antioxidant, anticarcinogenic, antidiabetic, antimicrobial, and immunomodulatory properties [1–3]. Mushrooms' physiological properties have garnered attention as a source of medicine and health supplements [4,5]. Species in the genus *Trametes* (*Basidiomycota*, *Polyporales*) are the white rot fungi that cause white rot in trees and wood. Of the approximately 60 *Trametes* species known globally, around 10 species have been reported in Korea [6–8]. Previous studies suggest that mycelia or fruiting bodies of *Trametes* spp. contain a variety of macromolecular compounds with pharmacological properties, such as antioxidant, anti-inflammatory, anticancer, and antimicrobial activities [9–12]. However, even in the well-categorized review, it is a challenge to compare the results from different studies because each study utilized different methods in selecting strains and media and measuring the activity [12]. The use of fruiting body extracts is common, but securing a stable supply of similar fruiting bodies can be difficult. An additional problem with the genus *Trametes* is the conflicting concepts surrounding its delimitation based on diagnostic morphological characters [13]. Recently, a phylogenetic study using five gene sequences revealed the taxonomic positions of the genus *Trametes*, finding that the five markers were sufficient to distinguish the genus *Trametes* from the other genera [13].

In this study, the physiological activities, with a focus on anticancer and antibacterial properties, of 19 strains of the genus *Trametes* from Korea were compared. The taxonomic positions of the strains were determined through a phylogenetic analysis using five gene sequences. Three culture media of each strain were used for extraction, allowing for comparison of activities among them. Large-scale comparative analysis in the genus *Trametes* showed that many strains possess medicinal properties with the potential for drug development.

2. Materials and methods

2.1. Fungal strains

This study used 19 strains from seven *Trametes* species, all sourced from Korea Mushroom Resource

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Table 1. *Trametes* strains used in this study.

			GenBank Accessions of Markers				
ID	Species	KMRB Accession	ITS	LSU	RPB1	RPB2	TEF1
1	*Trametes* cf. *junipericola*	17121423	ON402811	ON402832	OP216073	OQ167995	ON437541
2	*T.* cf. *junipericola*	14110701	ON402812	ON402833	OP216074	OQ167997	ON437542
3	*T.* cf. *junipericola*	16052615	ON402813	ON402834	OP216075	OQ167996	ON437543
4	*T. gibbosa*	17111316	ON402814	ON402835	OP216064	OQ167984	ON437544
5	*T. gibbosa*	16071413	ON402815	ON402836	OP216065	OQ167983	ON437545
6	*T. gibbosa*	15090213	ON402816	ON402837	OP216066	OQ167985	ON437546
7	*T. hirsuta*	18101711	ON402817	ON402838	OP216068	OQ167987	ON437547
8	*T. hirsuta*	18100832	ON402818	ON402839	OP216067	OQ167986	ON437548
9	*T. hirsuta*	17121406	ON402819	ON402840	OP216069	OQ167988	ON437549
10	*T. orientalis*	18091103	ON402820	ON402841	OP216061	OQ167981	ON437550
11	*T. orientalis*	17091716	ON402821	ON402842	OP216062	OQ167980	ON437551
12	*T. orientalis*	17111312	ON402822	ON402843	OP216063	OQ167982	ON437552
13	*T. suaveolens*	17121435	ON402823	ON402844	OP216077	OQ167993	ON437553
14	*T. suaveolens*	14101203	ON402824	ON402845	OP216078	OQ167994	ON437554
15	*T. suaveolens*	15052220	ON402825	ON402846	OP216076	OQ167992	ON437555
16	*T. trogii* (=*Coriolopsis trogii*)	15052235	ON402826	ON402847	OP216060	OQ167979	ON437556
17	*T. versicolor*	18111321	ON402827	ON402848	OP216071	OQ167990	ON437557
18	*T. versicolor*	18102611	ON402828	ON402849	OP216072	OQ167991	ON437558
19	*T. versicolor*	18101611	ON402829	ON402850	OP216070	OQ167989	ON437559

Table 2. Information of the five markers.

Markers	Primer names	Sequences (5 → 3)	References
RPB1	RPB1-Af	GARTGYCCDGGDCAYTTYGG	[27,28]
	RPB1-Cr	CCNGCDATNTCRTTRTCCATRTA	
RPB2-1	RPB2-f5F	GAYGAYMGWGATCAYTTYGG	[29,30]
	RPB2-b7.1R	CCCATRGCYTGYTTMCCCATDGC	
RPB2-2	RPB2 b6.9F	GCYCCYGGHCAYCGTGAYTTYAT	[31]
	RPB2 b11R1	CCRACRGCRACRGTYYGTCTCAT	
TEF1	EF1-983F	GCYCCYGGHCAYCGTGAYTTYAT	[32]
	EF1-2212R	CCRACRGCRACRGTYYGTCTCAT	
LSU	LR0R	ACCCGCTGAACTTAAGC	Vilgalys Lab
	LR7	TACTACCACCAAGATCT	
ITS	ITS1	TCCGTAGGTGAACCTGCGG	[33,34]
	ITS4	TCCTCCGCTTA TTGATATGC	

Bank (KMRB), Seoul National University. The detailed information about the samples is listed in Table 1. Strains were maintained at 25 °C in dark conditions on potato dextrose agar media (BD Difco 29 g in 1 L of water).

2.2. Sample preparation and PCR condition

Genomic DNA of 19 strains was extracted from homogenized mycelia of the 7-day-old samples using Nucleospin® Plant II kit from Macherey-Nagel company according to the manufacturer's instruction for fungi. The DNA quality was examined using the NanoDrop™ 2000 spectrophotometer. Amplification regions and used primers for PCR and sequencing are the same as those used in Justo and Hibbett [13]. The information on and amplified region and primer is listed in Table 2. Phire Hot Start II DNA Polymerase (Thermo Scientific™) was used in all of the PCR process. For RPB1 and RPB2-1, RPB2-2 region, the following "touchdown" PCR condition was used: (1) initial denaturation at 98 °C for 30 sec, (2) denaturation at 98 °C for 40 sec, (3) annealing at 60 °C for 40 sec (minus 1 °C per cycle), (4) extension at 72 °C for 2 min (5) repeat for 4 cycles starting at step2, (6) denaturation at 98 °C for 45 sec, (7) annealing at 57 °C for 1 min 30 sec, (8) extension at 72 °C for 2 min, (9) repeat for 36 cycles starting at step6, (10) leave at 72 °C for 1 min. LSU region was amplified according to the following process: (1) initial denaturation at 98 °C for 30 sec, (2) denaturation at 98 °C for 10 sec, (3) annealing at 98 °C for 5 sec, (4) extension at 98 °C for 25 sec (5) repeat for 34 cycles starting at step2, (6) leave at 72 °C for 1 min.

All PCR products were purified using AccuPrep® PCR/Gel Purification Kit according to manufacturer's instructions for PCR products. All purified products were sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kits. Additional sequence data of ITS, LSU, RPB1, RPB2, and TEF1 were downloaded from the NCBI database and included to phylogenetic analysis for comparison: 16 *Trametes*, 16 *Polyporales*, and one outgroup species (Supplementary Table 1).

2.3. Phylogenetic analysis

All acquired sequences were sorted and concatenated in the order of LSU, ITS, RPB1, RPB2, TEF1 using a SeqKit tool [14]. The concatenated sequences were aligned using the Muscle tool [15]. Phylogenetic trees were generated with by the RAxML v. 8.2 [16] and visualized in the R package 'GGTREE' [17]. Maximum likelihood analysis was performed in the RAxML under a GTRGAMMA substitution model with 1000 bootstrap replicates.

2.4. Preparation of extract samples for bioactivity measurement

A total of 13 strains belonging to the seven species were used for bioactivity tests. Those strains had been cultured for 7 days on PDA were cut into

circular pieces using a 5-mm cork borer. Ten plugs were inoculated to each Dextrose yeast extract (DY), Malt yeast extract (MY), and Malt extract (ME) broth and cultured for 30 days at the condition of 28 °C and 170 rpm. The cultured filtrates were separated from mycelium by using a sterile gauze and glass filter (porosity 100 ~ 160 µm, Duran) and then freeze-dried [9]. For the antibacterial activity test, freeze-dried samples were dissolved in dimethyl sulfoxide (DMSO) at 150 mg/mL. For anticancer activity test, the freeze-dried samples were extracted with 100% ethanol for 48 h in the rolling shaker at 50 rpm and then filtrated using a 0.45 um syringe filter. Ethanol was removed by evaporation and then samples were freeze-dried.

2.5. Antibacterial activity test

Escherichia coli (KCCM40271), *Pseudomonas aeruginosa* (KCCM11802), *Staphylococcus aureus* subsp. *aureus* (KCCM11335) were retrieved from Korean Culture Center of Microorganisms (KCCM). *E. coli* was cultured in nutrient agar (BD Difco 23 g in 1 L water). *P. aeruginosa* and *S. aureus* subsp. *aureus* was cultured in Muller Hinton agar (BD Difco 38 g in 1 L of water). Three bacteria strains are initially prepared in solid media, then inoculated in liquid media overnight at 37 °C, 120 rpm. As a positive control, meropenem was dissolved in DMSO at the concentration of 10 ng/µL. 20 µL of the meropenem solution was dropped on a filter paper disk (diameter: 8 mm) and dried completely before usage. In the experimental group, 3 mg of each sample was spotted on a filter paper disk. In the second screening, the amount of the freeze-dried samples used per disk was varied and applied in three different amounts, 3, 6, and 9 mg. The DMSO spotted disk was used as a negative control. The antibacterial assay was carried out using the paper disk diffusion method [18]. The objected bacteria were inoculated in liquid culture. When the optical density of the objected bacteria reached 1.5, the pour-plate method was processed. The bacterial culture was added into warm nutrient/Muller Hinton agar and poured on the empty plates to make inoculated bacterial plates. The dried paper disks containing freeze-dried samples or meropenem were placed on the bacterial plates and incubated at 37 °C. After 18 h, the diameter of the halo around the paper disks was measured.

2.6. Anticancer activity test

Anticancer activity of 39 samples was measured on Michigan cancer foundation-7 (MCF-7) cell line. MCF-7 cells were cultured in 10% fetal bovine serum, DMEM (Hi Media, Mumbai, India) included in 1% antibiotic. The cells were seeded in 10^4 cells/well of a 96-well plate and incubated at 37 °C in 5% CO_2, pH 7.0 ~ 7.2 for 24 h. The freeze-dried extracts, dissolved in DMSO, were treated to MCF-7 cells at final concentration of 500 µg/mL and the cells were incubated for an additional 48 h. To test cell viability, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was used. The activity of the mitochondria in living cells was measured by quantifying the amount of changed tetrazolium salt that was converted to formazan crystal. Cisplatin was used as the positive control. After incubation, MTT dye was added 5 mg/mL in PBS and incubated at 37 °C in 5% CO_2, pH 7.0 ~ 7.2 for 4 h. The medium was removed and 200 uL/well of DMSO was added. The plate was read at 540 nm using a UV spectrophotometer after it was incubated with shaking for 30 min to dissolve the formazan.

3. Results

3.1. Phylogenetic analyses confirmed the taxonomic positions of the 19 sample strains

To determine the systematic positions of our sample strains (Table 1), we relied on a previous phylogenetic study that a phylogenetic tree based on combined data from five genetic regions: ribosomal markers (LSU and ITS) and protein-coding genes (RPB1, RPB2, and TEF1-alpha) [13]. We obtained the sequence information for five genes in 19 sample strains in our study. The length of the combined sequences varied from 5387 base pairs (bp) to 6394 bp, with an average length of 6277 bp. In addition, sequence data of 32 reference species from the previous study were included in our analysis (Supplementary Table 1). The concatenated sequences ranged from 5501 bp to 7,651 with an average length of 6441 bp. A maximum likelihood tree was generated, resulting that all 19 strains formed seven clades (Figure 1). The core polyporoid clade consists of three subclades: trametoid, polyporus, and dentocorticium clades. We found that 18 sample strains were included in the trametoid clade while one strain, *T. trogii*, belongs to the polyporus clade. *T. trogii* has a new genus name, *Coriolopsis trogii*, as a result of the previous phylogenetic study [13]. Twelve out of 18 strains which belong to four species (*T. versicolor*, *T. suaveolens*, *T. hirsuta*, and *T. gibbosa*) formed the same clade with the reference species in the trametoid clade. Three other strains (id: 1, 2, and 3) which had been identified as *T.* cf. *junipericola* formed its own clade in the phylogenetic tree (Figure 1). We could not compare *T.* cf. *junipericola* with *T. junipericola* in the five-gene tree, because the sequence data of *T. junipericola* were limited to only two genes, LSU and ITS, in the previous study [13]. Additional maximum likelihood

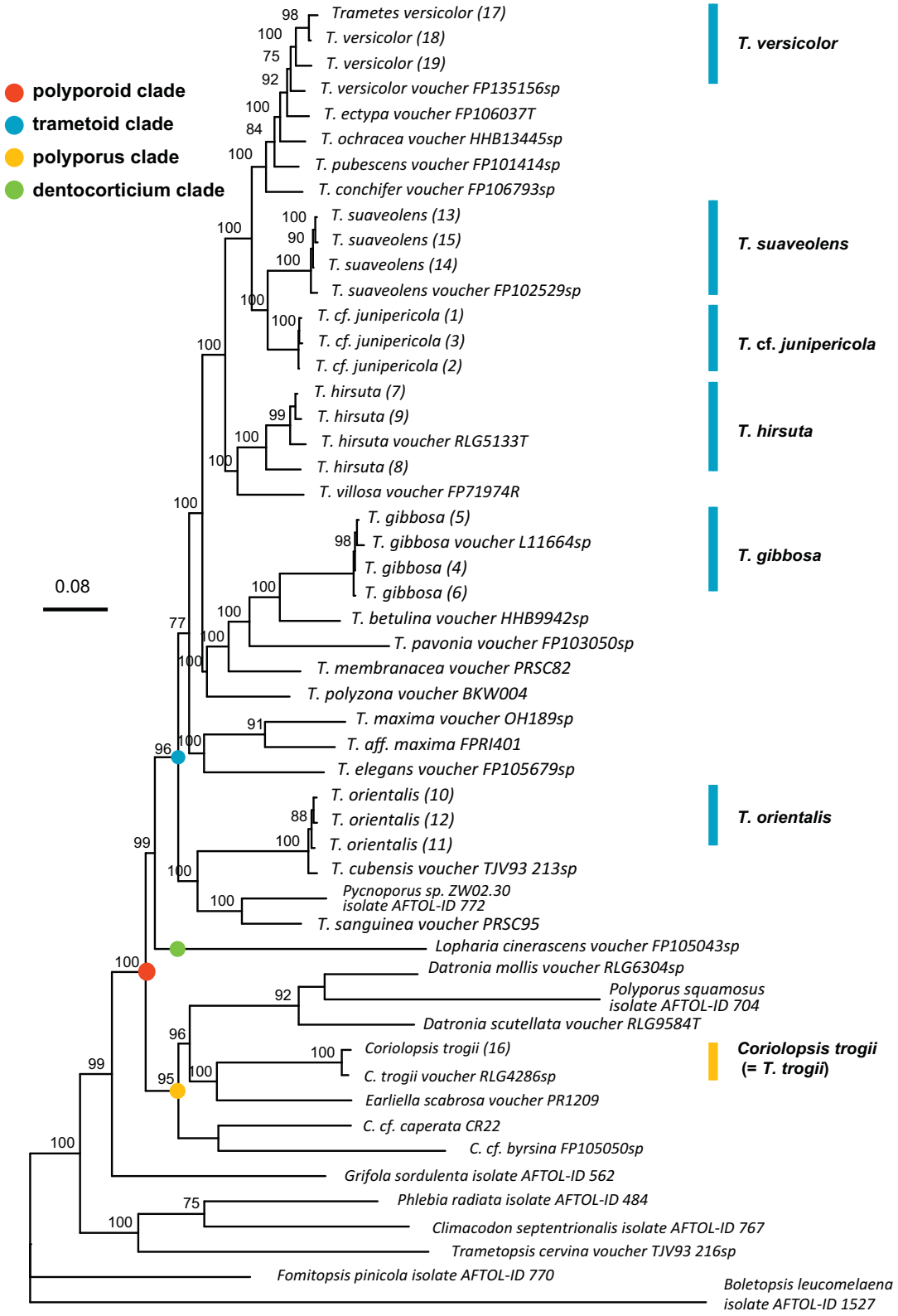

Figure 1. Phylogenetic relationships among *Trametes* species investigated based on the combined five marker dataset: ribosomal markers (LSU and ITS) and protein-coding genes (RPB1, RPB2, and TEF1). The number in bracket represents the identification number for the strains listed in Table 1. The colored circles indicate clades described previously [13].

tree was generated using concatenated sequences of the two genes from two strains of *T. junipericola* (Supplementary Fig. 1). The results indicated that the *T.* cf. *junipericola* strains formed a distinct clade separate from the *T. junipericola* strains, providing evidence that a new name is necessary for them. In addition, the other three strains (id:10, 11, and 12) which had been classified into *T. orientalis* located closely with *T. cubensis*. Similar to the *T.* cf. *junipericola* case, there was no *T. orientalis* strain had been included in the previous study [13]. We found four *T. orientalis* strains in the GenBank database but they have only four genes (except RPB2) to compare with our phylogenetic tree. Another maximum

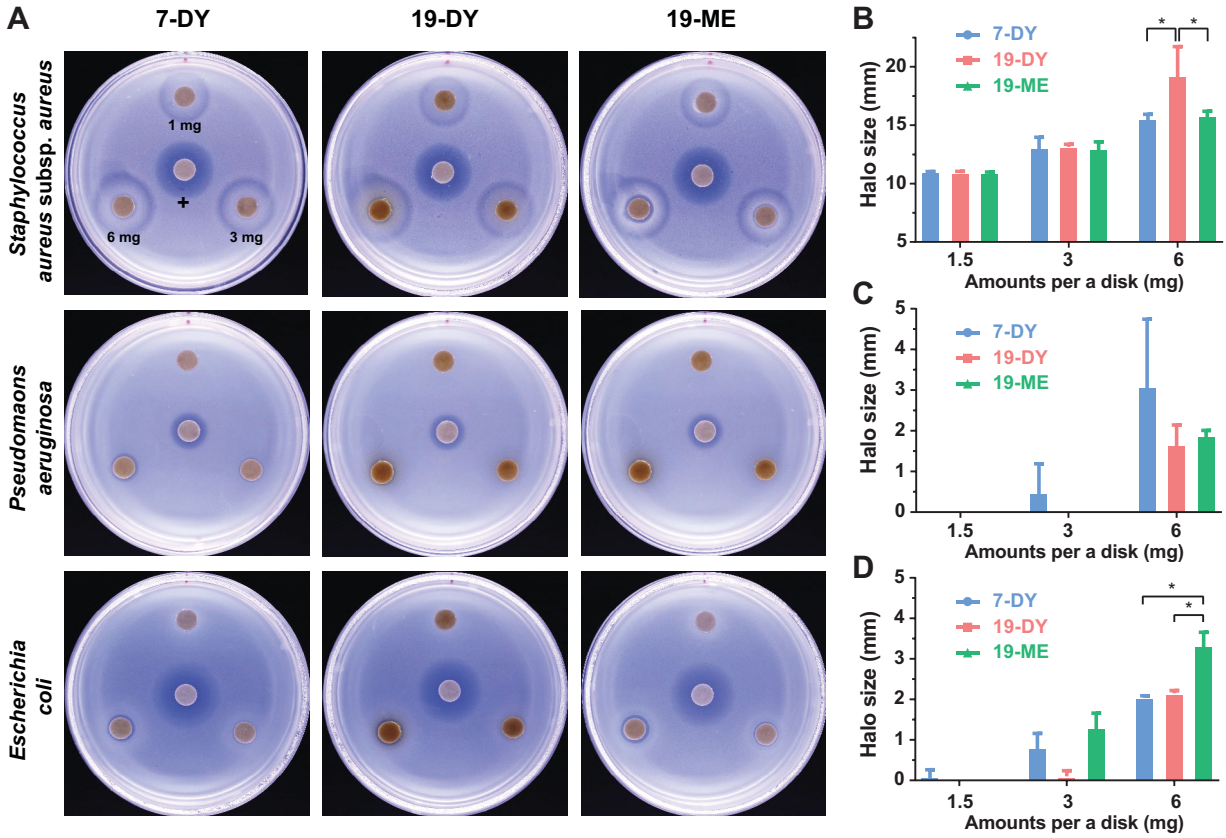

Figure 2. The antibacterial activity of *Trametes hirsuta* (7-DY) and *T. versicolor* (19-DY and 19-ME) on different bacteria. DY and ME are the media used for culture. 1.5 mg, 3 mg and 6 mg of each freeze-dried samples were added on each paper disk, then placed on the media containing *Staphylococcus aureus* subsp. *aureus* (B), *Pseudomonas aeruginosa* (C), and *Escherichia coli* (D). a positive control, meropenem, was placed at the center of each plate. Photos were taken after 18 h of incubation. Experiments were repeated twice. The halo size was measured by subtracting the size of the paper disks from its diameter. One-way ANOVA was applied to the data of '6 mg'. The opaque substances inside the halo are thought to be some diffused components of each extract.

likelihood tree constructed based on four genes revealed that our three *T. orientalis* strains formed a group that was phylogenetically similar to *T. cubensis* and the four other *T. orientalis* strains (Supplementary Fig. 2). This result suggests that more in-depth examination using additional genetic markers is necessary to clarify the classification within this species complex.

3.2. T. versicolor cultured on DY media (19-DY) showed the highest antibacterial activity on S. aureus subsp. aureus

To evaluate their antibacterial properties, 39 culture filtrates of 13 strains (two strains for six species and one for one species) were prepared and tested against three different bacteria (Supplementary Fig. 3). Among them, four samples, named as 7-DY, 7-ME, 19-DY and 19-ME, showed strong inhibition on *S. aureus* subsp. *aureus*. 7-DY, 19-DY and 19-ME samples were subjected to a second-round screening to determine their antibacterial activity. The 7-ME sample was excluded from further experiment due to the insufficient sample amount (Figure 2). As shown in Figure 2A, the higher concentration of the samples was used, the bigger the halo appeared on the bacteria-growing media. This result indicated that all three samples significantly inhibited the growth of *S. aureus* subsp. *aureus* in a concentration-dependent manner. The result of the second-round screening is equivalent with the first screening data (Supplementary Fig. S1). Especially, for *S. aureus* subsp. *aureus*, it is shown that the 19-DY caused a significantly bigger halo than other samples ($p < 0.05$, Figure 2B). The halo size made by '6 mg' of 19-DY was even bigger than that made by the positive control, meropenem (200 ng, Figure 2A). Meanwhile, the halo sizes of three samples on *P. aeruginosa* and *E. coli*, were less than 5 mm (Figure 2C and 2D, respectively). It could be concluded that the inhibition of three samples was effective specifically against the Gram-positive bacterium, *S. aureus* subsp. *aureus*.

3.3. Strong anticancer activity was found in various species of genus Trametes

The anticancer activity of the samples was assessed by measuring the cell viability of the cancer cell line MCF-7 in the presence of 39 different sample

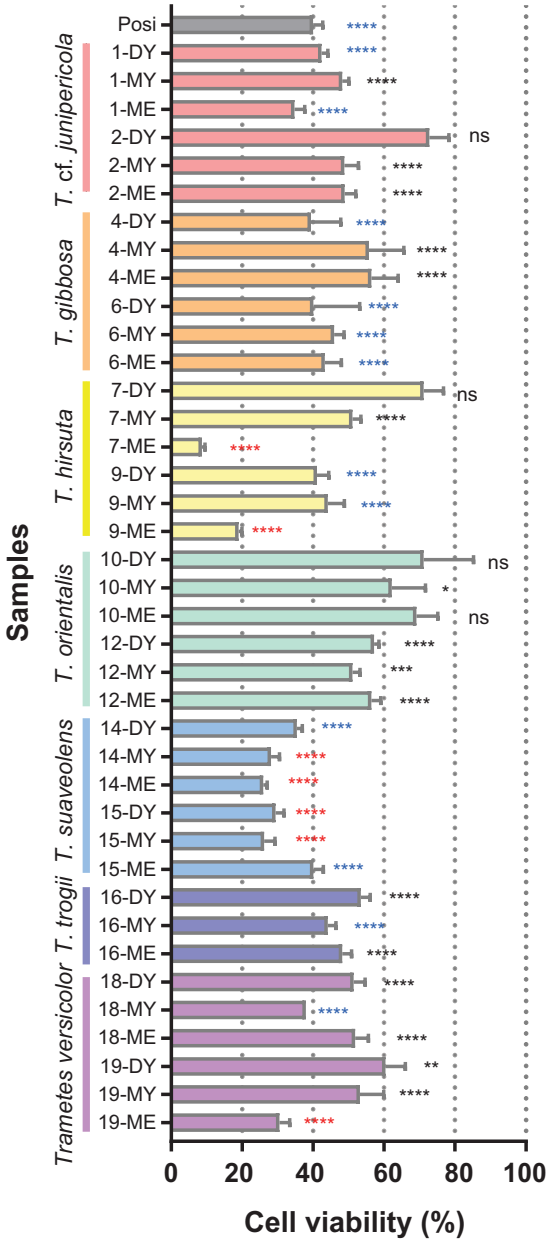

Figure 3. Anticancer activity of the culture extracts of 13 *Trametes* strains growing on different media. Cell viability was tested using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) on MCF-7 cells. The freeze-dried extracts were treated to the cells at 500 μg/mL as final concentration and incubated for the additional 48 h. Significance was determined in comparison with the negative control (media only) using one-way ANOVA with dunnet's multiple comparison (*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$, ns: non-significant). red and blue asterisks indicate stronger and similar performance, when compared to the positive control, respectively. Posi: 0.1 mM cisplatin was used as a positive control.

extracts (Figure 3). The lower cell viability observed, the stronger anticancer activity can be detected. One-way ANOVA test was performed with Dunnett's multiple comparisons to the positive control, 0.1 mM cisplatin. It is interesting to note that 35 out of 39 extracts showed significant anticancer activity compared to the negative control (media without fungal inoculum). Especially, seven samples with red asterisks showed even stronger anticancer activities than the positive control, 0.1 mM cisplatin (Figure 3). Among the seven samples, 7-ME and 9-ME were the strongest, indicating that the inhibition rates of 7-ME and 9-ME were 92.3% and 81.0%, respectively. Both of them were extracted from the same ME broth media of the same species, *T. hirsuta*. The extracts of 14-MY, 14-ME, 15-MY, and 15-DY among the seven samples, were from the same species, *T. suaveolens*, supporting the existence of the anticancer activities. It was found that 12 samples with blue asterisks showed statistically similar activities to the positive control. They belong to all species except one, *T. orientalis*. Four of them (4-DY, 6-DY, 6-MY, and 6-ME) came from *T. gibbosa* while two were extracted from the cultures (1-DY and 1-ME) of *T.* cf. *junipericola*. Two extracts (9-DY and 9-MY) of *T. hirsuta* also included in this group, supporting its consistent activity regardless of culture media.

4. Discussion

Phylogenetic analysis of 19 *Trametes* strains revealed that the 18 strains belong to genus *Trametes* and one belong to newly named sister genus, *Coriolopsis*. Based on the results of the physiological activity tests, two species, *T. hirsuta* and *T. versicolor*, expressed the strong antibacterial activity against *S. aureus* subsp. *aureus*. In addition, the extracts of *T. hirsuta*, *T. suaveolens*, and *T. versicolor* showed strong anticancer activity against the MCF-7 cells.

Some species in the genus *Trametes* have been known as having anticancer potential to treat various types of cancer [12]. *T. versicolor*, called as "Yun-Zhi" in China, is the most studied species. For example, 11 out of 15 studies in the review included this species [12]. Other studies have reported anticancer activities of *T. hirsuta* [19,20], *T. robiniophila* [21], *T. lactinea* [22], *T. gibbosa* [20], *C. trogii* [23]. Therefore, we reported anticancer activities of *T. suaveolens* and *T.* cf. *junipericola* for the first time. Unlike other studies using mycelial extracts, the extracts of culture filtrates were used in this study. This study is also the first report of effectiveness of culture filtrates from *T. hirsuta*, *T. gibbosa*, and *C. trogii*.

The experimental strategy, which involves the use of two strains from one species and three different cultures were used for extraction, is believed to made the results of this study reliable. Especially, all strains and cultures of *T. suaveolens* showed strong anticancer activity, supporting that *T. suaveolens* is a promising candidate for the development of anticancer drugs. The choice of culture media for extraction was found to be important. The 7-DY extract did not show a significant effect, while the

7-ME extract showed an inhibition rate of 92.3%. A similar trend was also observed in four other strains (id: 2, 9, 14, and 19).

This study confirmed the presence of anticancer activity in most strains, but only found antibacterial activity in two species, *T. hirsuta* and *T. versicolor*. The activity was only shown in one of the two strains of each species (*T. hirsuta*: 7-DY, 7-ME and *T. versicolor*: 19-DY, 19-ME), indicating that every individual strain can exhibit different activities even within a same species (Figure 2). Hleba et al. (2014) reported that the methanol extract of *T. versicolor* showed antibacterial activity against four bacterial species [24]. As in our study, it was not active against Gram-negative bacteria, *E. coli* and *P. aeruginosa*, but showed activity against Gram-positive bacteria, *S. epidermis* and *Enterococcus raffinosus* [24]. However, the extracts used in the study of Janes et al. had no effect on Gram-positive bacteria, *S. aureus* and *En. faecalis* but weak activity on Gram-negative *P. aeruginosa* [25]. The ethanol extracts of *T. hirsuta* exhibited wide activities against both Gram-positive and negative bacteria [26]. The lack of common patterns from the previous studies suggests that the antibacterial activity is specific to each strain.

In conclusion, we examined antibacterial and anticancer activities among 13 strains belong to the genera *Trametes* and *Coriolopsis*. The study confirmed the presence of anticancer activities in 10 strains belong to 6 species and antibacterial activities in 2 strains belong to 2 species. Especially, anticancer activities of *T. suaveolens* and *T.* cf. *junipericola* were reported for the first time. These results suggested that the genus *Trametes* is a potential source for the development of a new anticancer drug. Further experiments will be needed to identify the specific active compounds in the candidate strains.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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