Research Article

Antimicrobial Activities and Synergistic Effects of the Combination of some Edible Mushroom Extracts with Antibiotics against Pathogenic Strains

Sirikhwan Tinrat*

[1]Dept of Biotechnology, Faculty of Applied science, King Mongkut's University of Technology, North Bangkok, Bangkok, Thailand.

***Corresponding author's E-mail:** sirikhwan.t@sci.kmutnb.ac.th

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ABSTRACT

The antibacterial activities and synergistic effects of five edible mushroom extracts with antibiotics were investigated by agar well diffusion assay, checkerboard dilution method and time-kill curve method. Ethanolic *Lentinus edodes* (Berk.) Sing and *Pleurotus eryngii* (DC.) Gillet extracts were showed the inhibition zone (IZ) against *S. aureus* ATCC 25923 with 9.50±0.00 mm (200 mg/ml) and *S. typhimurium* ATCC 13311 with 18.67±0.58 mm (400 mg/ml). For broth microdilution method, ethanolic *Flammulina velutipes* (Curt:Fr.) Singer (FE) extract had MIC values of 800 mg/ml in all tested strains and had the MBC values of 1,600 mg/ml against *P. aeruginosa* ATCC 9027. In the synergistic antibacterial effects, the interaction of FE extract (1/32 MIC) and amoxicillin (1/64 MIC) was significantly showed the highest synergistic activity (FICI = 0.05) against *B. cereus* ATCC 5040 and *P. mirabilis* DMST 8212 ($P<0.05$). The synergistic interaction between FE extracts (1/4 MIC) and ciprofloxacin (1/8 MIC) also had the inhibitory effect to *P. mirabilis* DMST 8212 (FICI = 0.38). PW (1/16 MIC) extract was showed synergistic effects with amoxicillin (1/30 MIC) against *E. faecalis* DMST 4736 (FICI = 0.05). Moreover, the time-kill curves of sample extracts also significantly indicated a synergic biocidal effect between FE extract and amoxicillin against the *B. cereus* ATCC 5040 and *P. mirabilis* DMST 8212. These findings indicated that PW or FE extracts and amoxicillin or ciprofloxacin not only showed good potential synergistic antibacterial effect against some pathogens but also led to choice of good alternative antimicrobial agents for the treatment of infectious diseases.

Keywords: Edible Mushroom extracts, Synergistic Effects, Antimicrobial activity, Antibiotics.

INTRODUCTION

Infectious diseases still represent an important cause of morbidity and mortality among humans, especially in developing countries. Even though pharmaceutical industries have produced a number of new antimicrobial drugs in the last years, resistance to these drugs by microorganisms has increased. Today, clinically important bacteria are characterized not only by single drug resistance, but also by multiple antibiotic resistance the legacy of past decades of antimicrobial use and misuse.[1] Antibiotics that work today may not work tomorrow. This has result to search the effective antimicrobial agents from alternative natural resources like plant products. The use of plant extracts with known antimicrobial properties can be of great significance in therapeutic treatments. Many plants have been used because of their antimicrobial traits, which are due to compounds synthesized in the secondary metabolism of the plant. These products are known by their active substances. The antimicrobial properties of plants have been investigated by a number of researchers but research on synergistic effects is very limited and few studies have been reported.[2-5] For a long time mushrooms have been playing an important role in several aspects of the human activity. Mushrooms have been recognized as functional foods and as a source for the development of medicines and nutraceuticals.[6-7] There is not an easy distinction between edible and medical mushrooms because many of the common edible species have therapeutic properties and several used for medical purposes are also edible.[8] Numerous mushroom extracts showed potential antimicrobial activity against the pathogenic organism due to the presence of bioactive compounds like terpenoids, alkaloids and phenol which exhibit antimicrobial activity.[9] The most cultivated mushroom worldwide is *Agaricus bisporus*, followed by *Lentinus edodes*, *Pleurotus* spp., and *Flammulina velutipes*.[10] Some mushroom extracts, including *Laetiporus sulphureus*[11-12], *Ganoderma lucidum*[13] and *Lentinus edodes*[14] have already demonstrated antibacterial activity. In recent years, the most human pathogenic microorganisms have developed multiple drug resistance mechanism, due to indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases. This situation has forced scientists to search for new antimicrobial substances from various sources of novel antimicrobial chemotherapeutic agents.[15-16]

Therefore, the present study has been carried out to find out an alternative medicine for controlling pathogenic bacteria by using combinations of some edible mushroom extracts and antibiotics.

MATERIALS AND METHODS

Collection of plant materials and antibiotics

A total of five edible mushroom materials were selected from local market and Big C Supermarket, Bangkok province, in the central region of Thailand.

All of them (Table 1) are commonly consumed in Thailand. Commercial antibiotic Ampicillin and Ciprofloxacin was used during antibacterial study.

Sample preparation and extraction

Five of edible mushrooms were collected and thoroughly cleaned with distilled water, and soaked with 70% alcohol for 15 min. Each edible mushrooms were separated and soaked in 95% ethanol, 75% acetone and sterilized water in a ratio of 1:4 at 5°C for 10 day, respectively. The mixtures were filtered through a filter paper (Whatman No. 1) and centrifuged at 8,000 rpm for 15 min. Then, the filtrates were subsequently concentrated under vacuum on a rotary evaporator. The concentrated extracts were stored at -20°C under dark condition until further analysis. The final weight of the crude extracts was weighted and calculated for the percentage yield.

Antimicrobial activities

Microorganisms and Culture condition

In-vitro antimicrobial activities of all sample extracts at different concentrations were determined by agar disc diffusion method and minimum inhibitory and bactericidal concentration (MIC and MBC) assay against nine pathogenic strains including Gram-positive bacteria (*Bacillus cereus* DMST 5040, *Enterococcus faecalis* DMST 4736, *Staphylococcus aureus* ATCC 1216 and *Staphylococcus epidermidis*) and Gram-Negative bacteria (*Escherichia coli* ATCC 25922, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* DMST 8212 and *Salmonella typhimurium* ATCC 13311) were obtained from the laboratory of the Department of Biotechnology, King Mongkut's University of Technology, North Bangkok, Thailand. All tested strains were maintained on brain heart infusion (BHI, Difco) agar medium at 37°C.

Agar well diffusion method

The antimicrobial activity of the mushroom extracts was carried out by agar well diffusion method[17] against nine indicator pathogenic strains. Overnight bacterial cultures of tested strains were adjusted the OD_{600} to 0.2 (10^8-10^9 cfu/ml) by spectrophotometer. Briefly, 25 ml of BHI Agar was poured into each petri plate. Once the agar solidified, the microorganisms were mixed into 0.75% BHI agar and poured on the surface of the plates (1×10^8 cfu/ml). Subsequently, the surface of the agar was punched with a 6-mm-diameter wells by using a sterile cork borer. Each well was filled with 50 µl of each mushroom extracts. The concentration of the extracts employed was 200, 400, 600, 800 mg/ml and concentrated extracts, respectively. Simultaneously, Ampicillin (Amp, 5 µg/ml) and Ciprofloxacin (CIP, 5 µg/ml) were used as positive control. After 1, 3, 5, and 7 day incubation at 37°C, all plates were observed for zones of growth inhibition, and the diameter of these zones was measured in millimeters.

Determination of the minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) values of plant extracts were determined for the bacterial strains sensitive to the mushroom extracts in the broth micro-dilution method. The different mushroom extracts were first dissolved in 10% DMSO at the highest concentration of 6,400 mg/ml. The two-fold serial dilutions of extracts were made in a concentration range from 6,400 to 12.5 mg/ml with BHI broth in 96 well plates. Then, 50 µl of a standard inoculum of the pathogenic strains was added to each concentration of edible mushroom extracts. Contents of each tube were vortexed for 20 sec and then incubated at 37°C for 24 h. Similar tests were performed simultaneously for growth control (BHI + inoculums) and sterility control (BHI + test sample). The tube with least concentration of extract without growth after 24 h of incubation was recorded as the minimum inhibitory concentration (MIC).

Determination of the Minimum Bactericidal Concentration (MBC)

After MIC determination, all tested tubes showing complete absence of tested strains growth were identified. One loop full of each tested tube was transferred on fresh BHI agar (Difco) plate. After overnight incubation at 37°C, the least concentration of the crude extract which showed the complete absence of growth on the agar plates was considered as the Minimum Bactericidal Concentration (MBC).

Synergistic antimicrobial effects

The synergism between mushroom extracts and commercial antibacterial drugs was investigated in 96-well microtiter plates by checkerboard dilution method. Two antibiotics including amoxicillin (AM) and ciprofloxacin (CIP) were used. Synergistic effects of the combinations were investigated in antibiotics (2xMIC) and each extracts (4xMIC). The interaction between the two antimicrobial agents was estimated by calculating the fractional inhibitory concentration index (FICI). The FIC of each compound was calculated by dividing the concentration of the plant extracts in effective MIC of the combination, with the MIC of the drug alone. FICI values were calculated as;

FICI = FIC(A) + FIC(B)

= [A] / MIC (A) + [B] / MIC (B)

[A] **:** MIC value of A in a mixture of A and B substance

[B] : MIC value of B in a mixture of A and B substance

MIC (A) : MIC values of A substance

MIC (B) : MIC values of B substance

FICI values were interpreted as follows: FICI ≤ 0.5 Synergy (S); 0.5 > FICI ≤ 1 Additive (AD); >1.0 < FICI ≤ 4.0 Indifference (no effect: I) and FICI > 4.0 Antagonism (A).[18-19] Each test was repeated three times.

Time-kill curves

In-vitro bactericidal activities were evaluated using time kill technique according to NCCLS (1999).[20-22] The bactericidal activities of between mushroom extracts and drugs at synergistic effects of combine concentrations

(FICI values) from this study were evaluated by using time-kill curves. Mixture containing the mushroom extract, antibiotic and tested bacteria were incubated at 37°C, and viable counts were conducted at 0, 3, 6, 9, 12, 15, 18, 21, 24, 28 and 32 h by plating aliquots of the samples on agar and subsequent incubation for 24 h at 37°C. Cultures with an initial cell density of 1.5-4.0×10^8 cfu/ml were exposed to the MBC of the combination of plant extract and ampicillin or ciprofloxacin. Curves were constructed by plotting the viable cells (log cfu/ml) versus time. Synergy was defined as ≥2 log10 decreases in CFU of organisms treated with the drug combination compared to each treatment alone and control (untreated).[23]

Statistics analysis

Results obtained were reported as mean ± standard deviation (SD) of triplicate measurements. Statistical analyses (ANOVA) were performed with the statistical program MS Excel (Microsoft Office 2010 Professional) to analyze whether there was significant difference between each extract. P-values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Antimicrobial activities of some edible mushroom extracts

In current study, five edible mushroom extracts in different solvent (95% ethanol, 75% acetone and distilled water) were used to carry out the antibacterial activity against nine pathogenic strains by agar well diffusion method. The antibacterial activity of the each edible mushrooms species were varied significantly among the various bacterial species (Table 2). Ethanolic extract of *Pleurotus sajor-caju* (Fr.) Singer (PE) was significantly showed antimicrobial activity against only two bacterial strains including *S. typhimurium* ATCC 13311 and *B. cereus* DMST 5040 that had inhibition zone of 18.67±1.53 mm (600 mg/ml) and 10.00±0.00 mm (800 mg/ml), respectively (P<0.5). The average of inhibition zone of the FE extract (10.00±0.00 mm) had more than ampicillin as standard (9.60±1.45 mm). In addition, PE extract had more average inhibition zone than Acetone extract of *Lentinus edodes* (Berk.) Sing. (10.33±0.58 mm), *Pleurotus eryngii* (DC.) Gillet (9.67±0.58 mm) and *Hypsizygus tessellatus* (7.00±0.00 mm) at concentration of 800 mg/ml could inhibit to *S. aureus* ATCC 1216, which is consistent with research that has found; *Pleurotus eryngii* (DC.) Gillet that had anti-*S. aureus* (12.0 ± 1.4 mm)[24], as a Gram-positive. In another study by Loknath (2014)[25] also investigated that the *Pleurotus sajor-caju* extract showed the maximum zone of inhibition against *Enterococcus* sp., *S. typhimurium* S. pyogenes, *B. subtilis*, *K. pneumoniae* and *E. coli.* And water extract of *Flammulina velutipes* (Curt:Fr.) Singer and *Lentinus edodes* (Berk.) Sing. at concentration of 400 mg/ml had slightly anti-*P. mirabilis* DMST 8212 and *E. coli* ATCC 25922 growth, with inhibition zone of 7.00±0.00 and 6.33±0.29 mm, respectively. However, this study showed that the ethanolic extracts of all mushrooms could inhibit the tested strains growth (except *E. faecalis* DMST 4736 that had inhibition zone range from 7.00 ± 0.00 to 11.6 ± 1.15 mm (Data not shown)). In case of *Flammulina velutipes* (Curt:Fr.) Singer and *Hypsizygus tessellatus*, acetone extract was more effective than water and ethanol extracts of edible mushrooms. *S. aureus* ATCC 1216 was found to be the most sensitive strain and *E. faecalis* DMST 4736, *S. epidermidis*, *K. pneumoniae* and *P. aeruginosa* ATCC 27853 were the most resistant strains against these edible mushroom extracts. Especially, *P. aeruginosa* ATCC 27853, which belong to the group of the most resistible bacterial strains. The ability of *P. aeruginosa* to produce extracellular polysaccharides increased antimicrobial resistance.

The growth inhibitory effects of the edible mushroom extracts by broth micro-dilution method were presented in Table 3. The result indicated that the mushroom extracts were presented antibacterial activities at variable degree against tested pathogenic strains, with MIC values varying from 800 to more than 3,200 mg/ml. The MIC analysis of plant extracts showed the optimum bacteriostatic concentration for ethanolic crude extracts of the tested mushrooms. The ethanolic extract of *Flammulina velutipes* (Curt:Fr.) Singer and *Hypsizygus tessellatus* were exhibited antibacterial effects against all of the tested bacterial strains with MIC values of 800 and 1,600 mg/ml, respectively. While the water extract of *Pleurotus sajor-caju* (Fr.) Singer had MIC values of 3,200 mg/ml against all of the tested bacterial strains. The MBC value of all crude extract of mushrooms were >3,200 mg/ml except the ethanolic *Flammulina velutipes* (Curt:Fr.) Singer extract that had MBC values of 1,600 mg/ml against *P. aeruginosa* ATCC 27853. On the basis of MIC and MBC values, *P. aeruginosa* ATCC 27853 showed the highest sensitivity.

Synergistic antimicrobial effects

Five crude extracts of tested mushrooms including water extracts of *Pleurotus sajor-caju* (Fr.) Singer (PW) and *Pleurotus eryngii* (DC.) Gille (PEW) and ethanolic extracts of *Pleurotus eryngii* (DC.) Gillet (PEE), *Hypsizygus tessellatus* (HE) and *Flammulina velutipes* (Curt:Fr.) Singer (FE) were studied synergistic antibacterial effects due to showing MIC and MBC values against tested pathogens in broth microdilution method. The synergistic effects of edible mushroom extracts administered in combination with amoxicillin and/or ciprofloxacin are shown in Table 4 and 5, respectively. The Fractional Inhibition Concentration Index (FICI) obtained from the checkerboard dilution method. The result showed that the interactions between amoxicillin/ciprofloxacin and crudes extracts were synergistic, addition and indifference but were not antagonistic. When administered in combination with water and ethanol extracts, the MIC values of ampicillin and ciprofloxacin were reduced ≥ 8-120 and ≥8 folds to against all of

bacterial strains after testing. These findings indicated that a synergistic antibacterial effect base on a FICI of 0.028-0.38 and 0.38, respectively.

The antibacterial activities of combination between PW extract and amoxicillin were showed synergistic effect against all bacterial strains including *B. cereus* DMST 5040, *E. faecalis* DMST 4736, *S. aureus* ATCC 1216, *S. epidermidis*, *E. coli* ATCC 25922 and *K. pneumoniae*, *P. mirabilis* DMST 8212, *P. aeruginosa* ATCC 27853 and *S. typhimurium* ATCC 13311 while that of combination between PEW/HE extracts and amoxicillin had synergistic effect against six tested bacterial strains and indifference against approximately three tested strains. And interaction with FE extract (1/4 MIC) and amoxicillin (1/8 MIC) was also presented the synergistic effect (FICI = 0.380) against *P. aeruginosa* ATCC 27853 which is consistent with the result of antibacterial activity by broth microdilution method. And when combination of ciprofloxacin (1/8 MIC), the HE (1/4 MIC; FICI = 0.38) and FE (1/4 MIC; FICI = 0.38) extract had synergistic effects against five tested bacterial strains including *B. cereus* DMST 5040, *E. faecalis* DMST 4736, *S. aureus* ATCC 1216, *E. coli* ATCC 25922 and *K. pneumoniae* for HE extract and *B. cereus* DMST 5040, *E. faecalis* DMST 4736, *S. epidermidis*, *K. pneumoniae* and *P. mirabilis* DMST 8212 for FE extract.

When the treatment of urinary tract infection (*E. coli*, *E. faecalis, P. mirabilis*, *K. pneumoniae* and other strains), amoxicillin or ciprofloxacin were also one choice of the First-line antibiotics for therapy. The interaction with ethanolic extract of *Flammulina velutipes* (Curt:Fr.) Singer (FE) and both antibiotics (amoxicillin/ciprofloxacin) was showed synergistic effects to *E. faecalis* DMST 4736, *K. pneumoniae* and *P. mirabilis* DMST 8212 (FICI = 0.028-0.38) growth as urinary tract pathogens in this study. Moreover, the combination between water extract of *Pleurotus sajor-caju* (Fr.) Singer (PW) and amoxicillin also had synergistic effects against six tested bacterial strains and 4 out of 6 bacterial strains were E. *faecalis* DMST 4736, *S. aureus* ATCC 1216, *E. coli* ATCC 25922 and *K. pneumoniae* which were urinary tract infection. Only PW extract (1/4 MIC) that had a synergistic effect interaction with both amoxicillin (1/8 MIC) and ciprofloxacin (1/8 MIC) against *E. coli* ATCC 25922. On the other hand, the interaction of ethanolic *Hypsizygus tessellatus* (HE) extract and ciprofloxacin had absence of synergistic effects against *S. epidermidis* and *P. mirabilis* DMST 8212 (Table 5). These findings indicated that the combination between PW or FE extract and amoxicillin or ciprofloxacin was the best synergistic antibacterial effect for the treatment of common urinary tract infection in the future.

Time killing curve assay

The synergistic effects of the PW or FE extracts with amoxicillin and FE with ciprofloxacin at concentration of synergistic effects from checkerboard dilution method were confirmed by time killing curve assay.

The time kill curve of combination between plant extracts and antibiotics comparing with each treatment alone and control were shown in Figure 1-2. Cultures of each bacterial strains (*E. faecalis* DMST 4736 *S. epidermidis*, *B. cereus* ATCC 11778 and *P. mirabilis* DMST 8212) at a cell density of 10^8 cfu/ml were used for testing.

The result significantly showed that the growth of the *B. cereus* ATCC 5040 and *P. mirabilis* DMST 8212 were completely attenuated after 3.0 and 6.0 h at the FE extract : amoxicillin concentration ratios of 25 (1/32 MIC) : 0.0049 (1/64 MIC) mg/ml when comparing with each extract or medicine alone and control. ($P<0.05$) (Figure 1A - 1B). These combination had a potential synergic biocidal effect in 0 log CFU/ml of the inoculums after previous incubating. The interaction of PW extract (1/16 and 1/32 MIC) and amoxicillin (1/32 and 1/64 MIC) was able to inhibit and *S. epidermidis* and *E. faecalis* DMST 4736 in the first 6 and 15 h of incubation, which were growth reduction of least 2 log cfu/ml (Figure 1 C-D). While regrowth was found in only *E. faecalis* DMST 4736 incubated with the combination of PW extract (1/16 MIC) and amoxicillin (1/30 MIC) (FICI =0.090) about >1.5 log CFU/ml after 12 h of incubation.

Table 1: Description of 5 edible mushroom samples

Scientific name	Family	Common name	Part used
***Pleurotus sajor-caju* (Fr.) Singers**	Pleurtaceae	Grey oyster mushroom	Flowers and stalks
Hypsizygus tessellatus	Trichlomataceae	Buna-shimeji	Flowers and stalks
***Lentinus edodes* (Berk) Singer**	Pleurtaceae	Shiitake/Black mushroom	Flowers and stalks
***Flammulina velutipes* (Curt:Fr.) Singer**	Trichlomataceae	Golden needle mushroom	Flowers and stalks
***Pleurotus eryngii* (DC.) Gillet**	Pleurtaceae	The King Oyster Mushroom	Flowers and stalks

Table 2: The inhibition zone of mushroom extracts against some pathogens by agar well diffusion assay

Plant species	Concentration (mg/ml)		Average Inhibition Zone (AIZ); mm±SD								
			Gram positive strains				Gram negative strains				
			BC	EF	SA	SE	EC	KP	PA	PM	ST
Pleurotus sajor-caju (Fr.) Singer	Water	400	R	R	R	R	R	R	R	R	R
		600	R	R	R	R	R	R	R	R	R
		800	R	R	R	R	R	R	R	R	R
	Ethanol	400	R	R	R	R	R	R	R	R	R
		600	R	R	R	R	R	R	R	R	18.67±0.58[a]
		800	10.00±0.00[a]	R	R	R	R	R	R	R	18.67±0.58[a]
	Acetone	400	R	R	R	R	R	R	R	R	R
		600	R	R	R	R	R	R	R	R	R
		800	R	R	R	R	R	R	R	R	R
Lentinus edodes (Berk.) Sing.	Water	400	R	R	R	R	R	R	R	6.33±0.58[a]	R
		600	R	R	R	R	R	R	R	R	R
		800	R	R	R	R	R	R	R	R	R
	Ethanol	400	R	R	9.50±0.00[b]	R	R	R	R	R	R
		600	R	R	R	R	R	R	R	R	R
		800	R	R	R	R	R	R	R	R	R
	Acetone	400	R	R	R	R	R	R	R	R	R
		600	R	R	7.00±0.00[c]	R	6.67±0.58[b]	R	R	R	R
		800	R	R	10.33±0.58[a]	R	7.33[a]	R	R	R	R
Flammulina velutipes (Curt:Fr.) Singer	Water	400	R	R	R	R	7.00±0.00[ab]	R	R	R	R
		600	R	R	R	R	7.00±0.00[ab]	R	R	R	R
		800	R	R	R	R	7.00±0.00[ab]	R	R	R	R
	Ethanol	400	R	R	R	R	R	R	R	R	R
		600	R	R	R	R	R	R	R	R	R
		800	7.00±0.00[b]	R	R	R	R	R	R	R	R
	Acetone	400	R	R	R	R	R	R	R	R	R
		600	R	R	R	R	R	R	R	R	R
		800	R	R	R	R	R	R	R	R	R
Pleurotus eryngii (DC.)Gillet	Water	400	R	R	R	R	R	R	R	R	R
		600	R	R	R	R	R	R	R	R	R
		800	R	R	R	R	R	R	R	R	R
	Ethanol	400	R	R	R	R	R	R	R	R	R
		600	R	R	R	R	R	R	R	R	R
		800	R	R	R	R	R	R	R	R	R
	Acetone	400	R	R	R	R	R	R	R	R	R
		600	R	R	R	R	R	R	R	R	R
		800	R	R	9.67±0.58[b]	R	R	R	R	R	R
Hypsizygus tessellatus	Water	400	R	R	R	R	R	R	R	R	R
		600	R	R	R	R	R	R	R	R	R
		800	R	R	R	R	R	R	R	R	R
	Ethanol	400	R	R	R	R	R	R	R	R	R
		600	R	R	R	R	R	R	R	R	R
		800	R	R	R	R	R	R	R	R	R
	Acetone	400	R	R	R	R	R	R	R	R	R
		600	R	R	R	R	R	R	R	R	R
		800	R	R	7.00±0.00[c]	R	R	R	R	R	R

BC = *Bacillus cereus* DMST 5040, EF = *Enterococcus faecalis* DMST 4736, SA = *Staphylococcus aureus* ATCC 1216 and SE = *Staphylococcus epidermidis*) and Gram-Negative bacteria (EC = *Escherichia coli* ATCC 25922, KP = *Klebsiella pneumoniae*, PM = *Proteus mirabilis* DMST 8212, PA = *Pseudomonas aeruginosa* ATCC 27853, ST = *Salmonella typhimurium* ATCC 13311 and R = Resistance, [abc]**:** means in the same row with different superscript were significantly different (P < 0.05) by one way ANOVA and paired T-test.

Table 3: The MIC and MBC of edible mushroom extracts against some pathogenic strains by broth microdilution method

Plant species	Concentration (mg/ml)		Microorganisms								
			Gram positive strains				Gram negative strains				
			BC	EF	SA	SE	EC	KP	PA	PM	ST
Pleurotus sajor-caju (Fr.) Singer	Water	MIC	3,200^{a}	3,200^{a}	3,200^{a}	3,200^{a}	3,200^{a}	3,200^{a}	3,200^{a}	3,200^{a}	3,200^{a}
		MBC	-	-	-	-	-	-	-	-	-
	Ethanol	MIC	-	-	-	-	-	-	-	-	-
		MBC	-	-	-	-	-	-	-	-	-
	Acetone	MIC	-	-	-	-	-	-	-	-	-
		MBC	-	-	-	-	-	-	-	-	-
Lentinus edodes (Berk.) Sing.	Water	MIC	-	-	-	-	-	-	-	-	-
		MBC	-	-	-	-	-	-	-	-	-
	Ethanol	MIC	-	-	-	-	-	-	-	-	-
		MBC	-	-	-	-	-	-	-	-	-
	Acetone	MIC	-	-	-	-	-	-	-	-	-
		MBC	-	-	-	-	-	-	-	-	-
Flammulina velutipes (Curt:Fr.) Singer	Water	MIC	-	-	-	-	-	-	-	-	-
		MBC	-	-	-	-	-	-	-	-	-
	Ethanol	MIC	800^{c}	800^{c}	800^{c}	800^{c}	800^{c}	800^{c}	800^{c}	800^{c}	800^{c}
		MBC	-	-	-	-	-	-	1,600$^{1/a}$	-	-
	Acetone	MIC	-	-	-	-	-	-	-	-	-
		MBC	-	-	-	-	-	-	-	-	-
Pleurotus eryngii (DC.)Gillet	Water	MIC	3,200^{a}	3,200^{a}	3,200^{a}	3,200^{a}	3,200^{a}	3,200^{a}	3,200^{a}	3,200^{a}	3,200^{a}
		MBC	-	-	-	-	-	-	-	-	-
	Ethanol	MIC	-	-	-	-	-	-	-	-	-
		MBC	-	-	-	-	-	-	-	-	-
	Acetone	MIC	-	-	-	-	-	-	-	-	-
		MBC	-	-	-	-	-	-	-	-	-
Hypsizygus tessellatus	Water	MIC	-	-	-	-	-	-	-	-	-
		MBC	-	-	-	-	-	-	-	-	-
	Ethanol	MIC	1,600^{b}	1,600^{b}	1,600^{b}	1,600^{b}	1,600^{b}	1,600^{b}	1,600^{b}	1,600^{b}	1,600^{b}
		MBC	-	-	-	-	-	-	-	-	-
	Acetone	MIC	-	-	-	-	-	-	-	-	-
		MBC	-	-	-	-	-	-	-	-	-
Amoxicillin		MIC	0.3125^{A}	0.0391^{D}	0.0728^{C}	0.1563^{B}	0.3125^{A}	0.1563^{B}	0.0728^{C}	0.3125^{A}	0.0728^{C}
		MBC	1.2500^{A}	1.2500^{A}	0.6250^{B}	2.5000^{A}	0.6250^{B}	0.6250^{B}	1.2500^{A}	1.2500^{A}	1.2500^{A}
Ciprofloxacin		MIC	0.3125^{A}	0.3125^{A}	0.3125^{A}	0.3125^{A}	0.3125^{A}	0.3125^{A}	0.0195^{B}	0.3125^{A}	0.0195^{B}
		MBC	2.5000^{B}	1.2500^{C}	2.5000^{B}	5.0000^{A}	2.5000^{B}	0.6250^{D}	1.2500^{C}	2.5000^{B}	2.5000^{B}

BC = *Bacillus cereus* DMST 5040, EF = *Enterococcus faecalis* DMST 4736, SA = *Staphylococcus aureus* ATCC 1216, SE = *Staphylococcus epidermidis*, EC = *Escherichia coli* ATCC 25922, KP = *Klebsiella pneumoniae*, PA = *Pseudomonas aeruginosa* ATCC 27853, PM = *Proteus mirabilis* DMST 8212 and ST = *Salmonella typhimurium* ATCC 13311; ND = Not detected (at concentrated extracts were not found MIC and MBD values); (-) = >3,200 mg/ml for extracts

^{abc} and $^{1/a}$ **:** means in the same row with different superscript were significantly different ($P < 0.05$) by one way ANOVA and paired T-test (Among plant extracts with MIC values and MBC value, respectively)

ABC **:** means in the same column with different superscript were significantly different ($P < 0.05$) by one way ANOVA and paired T-test (Antibiotics)

Table 4: Synergistic antimicrobial effects of between crude extracts and ampicillin

Microorganisms	Extract : Medicine		MIC values (mg/ml)		MIC value of combination (mg/ml) (4MIC : 2 MIC)		FICI value	Interpreted
B. cereus ATCC 11778	PW	: AM	3,200	: 0.3125	200	: 0.0098	0.093	S
	PEW	: AM	3,200	: 0.3125	800	: 0.0391	0.380	S
	HE	: AM	1,600	: 0.3125	50	: 0.0049	0.050	S
	FE	: AM	800	: 0.3125	25	: 0.0049	0.050	S
E. faecalis DMST 4736	PW	: AM	3,200	: 0.0391	100	: 0.0007	0.050	S
	PEW	: AM	3,200	: 0.0391	3,200	: 0.0196	1.500	I
	HE	: AM	1,600	: 0.0391	50	: 0.0007	0.050	S
	FE	: AM	800	: 0.0391	50	: 0.0013	0.090	S
S. aureus ATCC 25923	PW	: AM	3,200	: 0.0782	200	: 0.0025	0.095	S
	PEW	: AM	3,200	: 0.0782	400	: 0.0049	0.190	S
	HE	: AM	1,600	: 0.0782	3,200	: 0.0782	3.00	I
	FE	: AM	800	: 0.0782	200	: 0.0098	0.380	S
S. epidermidis	PW	: AM	3,200	: 0.1563	100	: 0.0025	0.050	S
	PEW	: AM	3,200	: 0.1563	100	: 0.0025	0.050	S
	PEE	: AM	3,200	: 0.1563	50	: 0.0013	0.028	S
	HE	: AM	1,600	: 0.1563	25	: 0.0013	0.028	S
	FE	: AM	800	: 0.1563	12.5	: 0.0013	0.028	S
E. coli ATCC 25922	PW	: AM	3,200	: 0.3125	800	: 0.0391	0.380	S
	PEW	: AM	3,200	: 0.3125	800	: 0.0391	0.380	S
	HE	: AM	1,600	: 0.3125	200	: 0.0196	0.188	S
	FE	: AM	800	: 0.3125	100	: 0.0196	0.185	S
K. pneumoniae	PW	: AM	3,200	: 0.1563	100	: 0.0025	0.050	S
	PEW	: AM	3,200	: 0.1563	400	: 0.0098	0.190	S
	HE	: AM	1,600	: 0.1563	50	: 0.0025	0.050	S
	FE	: AM	800	: 0.1563	12.5	: 0.0013	0.028	S
P. aeruginosa ATCC 9027	PW	: AM	3,200	: 0.0782	1,600	: 0.0196	0.750	AD
	PEW	: AM	3,200	: 0.0782	3,200	: 0.0391	1.500	I
	HE	: AM	1,600	: 0.0782	800	: 0.0196	0.750	AD
	FE	: AM	800	: 0.0782	200	: 0.0098	0.380	S
P. mirabilis DMST 8212	PW	: AM	3,200	: 0.3125	3,200	: 0.1563	1.500	I
	PEW	: AM	3,200	: 0.3125	3,200	: 0.1563	1.500	I
	HE	: AM	ND	: 0.3125	ND	: ND	ND	ND
	FE	: AM	800	: 0.3125	25	: 0.0049	0.050	S
S. typhimurium ATCC 13311	PW	: AM	3,200	: 0.0782	1,600	: 0.0196	0.750	AD
	PEW	: AM	3,200	: 0.0782	3,200	: 0.0391	1.500	I
	HE	: AM	1,600	: 0.0782	800	: 0.0196	0.750	AD
	FE	: AM	800	: 0.0782	200	: 0.0098	0.380	S

PW = *Pleurotus sajor-caju* (Fr.) Singer extract in water; PEW = *Pleurotus eryngii* (DC.) Gillet extract in water; PEE = *Pleurotus eryngii* (DC.) Gillet extract in ethanol; HE = *Hypsizygus tessellatus* extract in ethanol; FE = *Flammulina velutipes* (Curt:Fr.) Singer extract in ethanol; AM = Ampicillin; ND = Not detected

Table 5: Synergistic antimicrobial effects of between crude extracts and ciprofloxacin

Microorganisms	Extract : Medicine		MIC values (mg/ml)		MIC value of combination (mg/ml) (4MIC : 2 MIC)		FICI value	Interpreted
B. cereus ATCC 11778	PW	: CIP	3,200	: 0.3125	1,600	: 0.0782	0.75	AD
	PEW	: CIP	3,200	: 0.3125	3,200	: 0.1563	1.50	I
	HE	: CIP	1,600	: 0.3125	400	: 0.0391	0.38	S
	FE	: CIP	800	: 0.3125	200	: 0.0391	0.38	S
E. faecalis DMST 4736	PW	: CIP	3,200	: 0.3125	ND	: ND	ND	ND
	PEW	: CIP	3,200	: 0.3125	3,200	: 0.1563	1.50	I
	HE	: CIP	1,600	: 0.3125	400	: 0.0391	0.38	S
	FE	: CIP	800	: 0.3125	200	: 0.0391	0.38	S
S. aureus ATCC 25923	PW	: CIP	3,200	: 0.3125	1,600	: 0.0782	0.75	A
	PEW	: CIP	3,200	: 0.3125	3,200	: 0.1563	1.50	I
	HE	: CIP	1,600	: 0.3125	400	: 0.0391	0.38	S
	FE	: CIP	800	: 0.3125	800	: 0.1563	1.50	I
S. epidermidis	PW	: CIP	3,200	: 0.3125	ND	: ND	ND	ND
	PEW	: CIP	3,200	: 0.3125	6,400	: 0.3125	3.00	I
	PEE	: CIP	3,200	: 0.3125	800	: 0.0391	0.38	S
	HE	: CIP	1,600	: 0.3125	ND	: ND	ND	ND
	FE	: CIP	800	: 0.3125	200	: 0.0391	0.38	S
E. coli ATCC 25922	PW	: CIP	3,200	: 0.3125	800	: 0.0391	0.38	S
	PEW	: CIP	3,200	: 0.3125	3,200	: 0.1563	1.50	I
	HE	: CIP	1,600	: 0.3125	400	: 0.0391	0.38	S
	FE	: CIP	800	: 0.3125	800	: 0.1563	1.50	I
K. pneumoniae	PW	: CIP	3,200	: 0.3125	ND	: ND	ND	ND
	PEW	: CIP	3,200	: 0.3125	3,200	: 0.1563	1.50	I
	HE	: CIP	1,600	: 0.3125	400	: 0.0391	0.38	S
	FE	: CIP	800	: 0.3125	200	: 0.0391	0.38	S
P. aeruginosa ATCC 9027	PW	: CIP	3,200	: 0.0195	ND	: ND	ND	ND
	PEW	: CIP	3,200	: 0.0195	6,400	: 0.0195	3.00	I
	HE	: CIP	1,600	: 0.0195	3,200	: 0.0195	3.00	I
	FE	: CIP	800	: 0.0195	1,600	: 0.0195	3.00	I
P. mirabilis DMST 8212	PW	: CIP	3,200	: 0.3125	ND	: ND	ND	ND
	PEW	: CIP	3,200	: 0.3125	6,400	: 0.3125	3.00	I
	HE	: CIP	ND	: 0.3125	ND	: ND	ND	ND
	FE	: CIP	800	: 0.3125	200	: 0.0391	0.38	S
S. typhimurium ATCC 13311	PW	: CIP	3,200	: 0.0195	ND	: ND	ND	ND
	PEW	: CIP	3,200	: 0.0195	6,400	: 0.0195	3.00	I
	HE	: CIP	1,600	: 0.0195	3,200	: 0.0195	3.00	I
	FE	: CIP	800	: 0.0195	1,600	: 0.0195	3.00	I

PW = *Pleurotus sajor-caju* (Fr.) Singer extract in water; PEW = *Pleurotus eryngii* (DC.) Gillet extract in water; PEE = *Pleurotus eryngii* (DC.) Gillet extract in ethanol; HE = *Hypsizygus tessellatus* extract in ethanol; FE = *Flammulina velutipes* (Curt:Fr.) Singer extract in ethanol; CIP = Ciprofloxacin; ND = Not detected

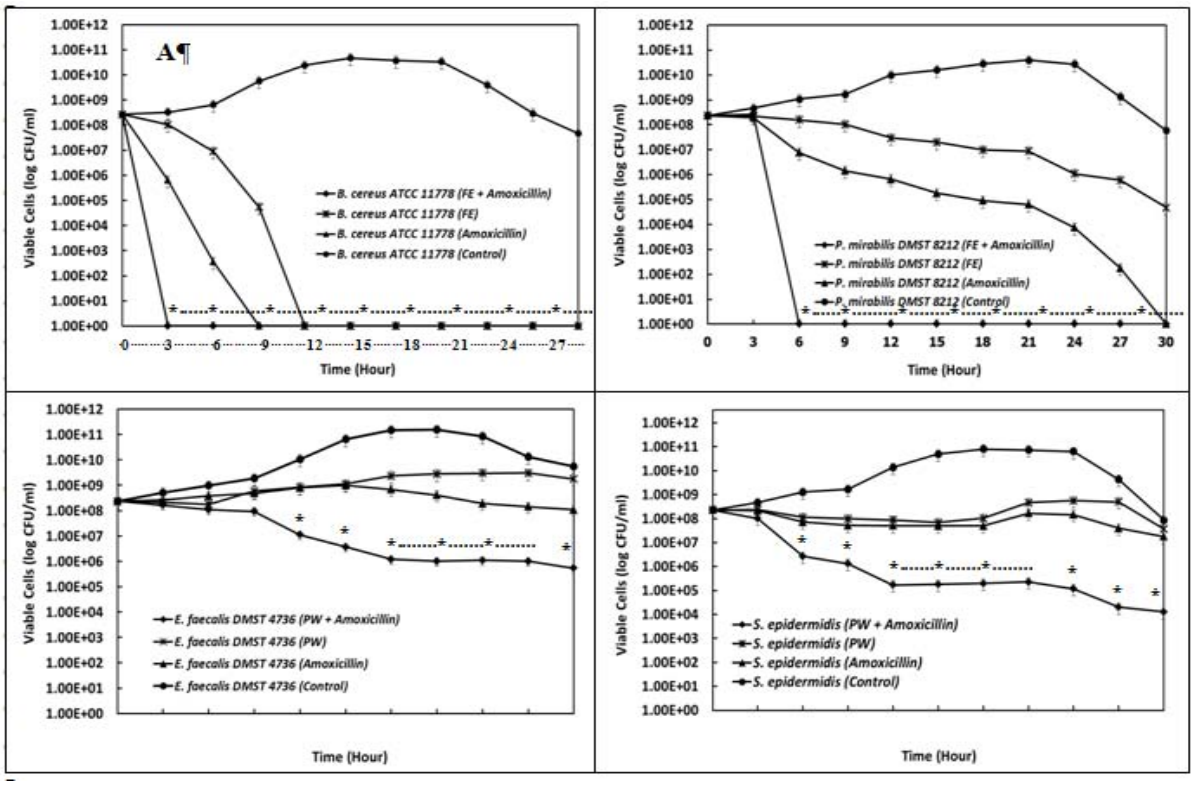

Figure 1: Time-kill curves of 1/32 MIC of *Flammulina velutipes* (Curt:Fr.) Singer in ethanol (FE) extract and (1/64 MIC) Amoxicillin alone its combination (FE : Amoxicillin; (1/32 MIC and 1/64 MIC)) against *B. cereus* ATCC 11778 (A), *P. mirabilis* DMST 8212 (B) and Time-kill curves of 1/16 MIC or 1/32 MIC of *Pleurotus sajor-caju* (Fr.) Singer in water extract (PW) and 1/32 MIC or 1/64 MIC Amoxicillin alone and its combination (FE : Amoxicillin) against *E. faecalis* DMST 4736 (1/32 MIC : 1/64 MIC) (C) and *S. epidermidis* (1/16 MIC : 1/32 MIC) (D) (Control = untreated; * = P<0.05 when comparing with control)

Moreover, the combination of FE extracts (1/4 MIC) and ciprofloxacin (1/8 MIC) had a significant synergic effect against *P. mirabilis* DMST 8212 in the first 12 h (growth reduction of 5 log CFU/ml) when compared control (10 log CFU/ml) (P<0.05) and each treatment alone (least 2 log) in the first 15 h (Figure 2) (P<0.05).

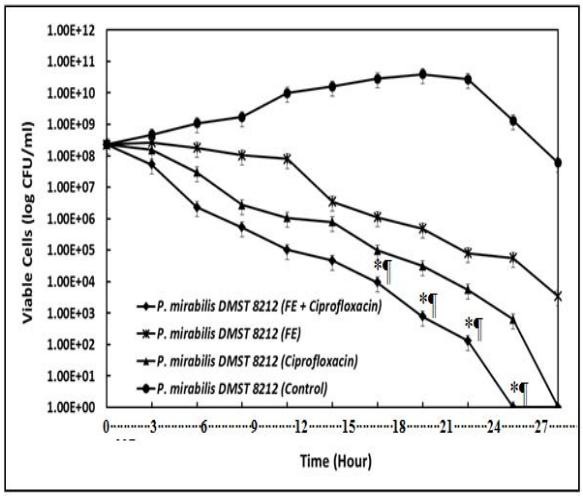

Figure 2: Time-kill curves of 1/4 MIC of *Flammulina velutipes* (Curt:Fr.) Singer in ethanol (FE) extract and 1/8 MIC Ciprofloxacin (1/8 MIC) alone and its combination (FE : Ciprofloxacin (1/8 MIC)) against *P. mirabilis* DMST 8212 (1/4MIC : 1/8 MIC) (Control = untreated; * = P<0.05 when comparing with control)

The present study demonstrated that the combination of FE extract (1/32 MIC) and amoxicillin (1/64 MIC) had the strong inhibitory effects against *B. cereus* ATCC 5040 and *P. mirabilis* DMST 8212 growth. And FE extracts (1/4 MIC) and ciprofloxacin (1/8 MIC) had inhibitory effect on *P. mirabilis* DMST 8212 growth. In other words, these combination had potential for the treatment and prevention of pathogenic infections especially urinary tract infection by *P. mirabilis* DMST 8212. Moreover, PW extract that showed potential synergistic effects with amoxicillin against *E. faecalis* DMST 4736, which can also cause urinary tract infection and other infections in humans. However, the synergistic effects from the association of different plant extracts and antibiotics against resistant bacteria lead to new choices for the treatment of infectious diseases. This effect enables the use of the respective plants when it is no longer effective by itself during therapeutic treatment.

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