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Evaluation of *Pseudomonas fluorescens* Extracts as Biocontrol Agents Against some Foodborne Microorganisms

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Abstract

Foodborne pathogens are among the serious problems in food industries. To control food spoilage microorganisms, five strains of *Pseudomonas fluorescens* were used as biocontrol agents against foodborne pathogenic bacteria and mycotoxigenic fungi. The antimicrobial activity of the *P. fluorescens* cell-free supernatants and their fractions were evaluated against six strains of foodborne pathogenic bacteria and five strains of toxigenic fungi. The bioactive fractions were identified using Gas Chromatography-Mass Spectroscopy (GC-MS). All cell- free supernatants of the *P. fluorescens* strains showed antibacterial activity against all tested bacteria with an inhibition zone ranging between 7.8 and 15.2 mm. While, only *P. fluorescens* FP10 exhibited antifungal activity against all tested fungi with a maximum inhibition zone of 19.3 mm, against *Aspergillus steynii*. The chloroform fractions of *P. fluorescens* FP6, FP7 and FP10 showed high activity against the tested pathogenic bacteria and toxigenic fungi. The GC-MS analysis of chloroform fractions of these strains showed the presence of two pyrrol compounds in all fractions as major compounds, Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- and Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(propylmethyl). The present study maintains that *P. fluorescens* cell-free supernatants and chloroform fractions would be ideal for application in food industry as biocontrol agents against foodborne pathogens.

Keywords: *Pseudomonas fluorescens*, Foodborne pathogens, Biocontrol, Mycotoxigenic fungi.

1. Introduction

Foodborne pathogens are among the major threats to food safety. Most foodborne illnesses occur by infections with pathogenic microbes that have contaminated the food chain at one or more points from farm to fork (Feltes *et al*., 2017). In addition, foodborne illnesses can be also caused by microbial toxins that contaminate food (Addis and Sisay, 2015). Mycotoxins are produced by certain fungal species as secondary metabolites that grow on several foods, including cereals, nuts and dried fruits. A variety of fungal species mostly from the genera *Aspergillus*, *Penicillium*, *Fusarium* and *Alternaria* are known to produce mycotoxins. Most important mycotoxins are aflatoxins, ochratoxin, fumonisins, deoxynivalenol, trichothecenes, zearalenone, and patulin (Karlovsky *et al*., 2016). Some strains of foodborne pathogenic bacteria including *Bacillus cereus*, *Staphylococcus aureus* and *Clostridium botulinum* produce toxins in food causing different symptoms ranging from vomiting in the case of the *S. aureus* toxin to high-risk neurological symptoms in the case of the botulinum toxin (Schirone *et al*., 2017).

Cooking and chilling are the major control measures to eliminate the foodborne microbes. Most foodborne diseases can be controlled by avoiding cross-contamination. This can be achieved by applying Good Manufacturing Practices (GMP) and Good Hygienic Practices (GHP) such as storing raw and cooked food separately and washing hands before and after touching raw food materials. Such food hygiene practices must be adhered to during production, storage, transportation and preparation of food, to minimize the growth and spread of pathogens (Malhotra *et al*., 2015). There are various methods that can be used to preserve food, including conventional methods such as drying, heating, freezing, fermentation, salting and modern preservation technology including biopreservative and active antimicrobial packaging systems (Sung *et al*., 2013; Darwesh *et al*., 2018).

Food biopreservation refers to the use of microorganisms or their metabolites to inhibit or destroy the undesirable microorganisms in foods to improve the microbiological safety and extend the shelf life of foods. Several studies have reported that use of microorganisms, such as bacteria, yeast, fungi, actinomycetes and algae and their antimicrobial metabolites as biopreservative substances (Marrez and Sultan, 2016; Sultan *et al*., 2016; Marrez *et al*., 2017). Some bacterial strains are able to produce antagonistic substances used as antimicrobial and biocontrol agents against microorganisms in food. In this respect, *Pseudomonas* spp. is one of the most considerable species used in the biocontrol of foodborne pathogenic bacteria and mycotoxigenic fungi (Sabry *et al*., 2016). *P. fluorescens* shows antagonistic effects against some toxigenic fungi such as *A. flavus*, *A. niger*, *P. italicum*, *P. simplicissmum* and *Fusarium* sp. (Srivastava and Shalini, 2008; Mushtaq *et al*., 2010). Moreover, it produces antibacterial agents against foodborne pathogenic bacteria *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Proteus mirabilis* (Kadhim, 2015). The main objective of

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this study is to evaluate the antagonistic potential of some *P. fluorescens* strains against foodborne pathogenic microorganisms. Furthermore, it aims at identifying the bioactive compounds of the most active fractions against foodborne pathogens using GC-MS.

2. Materials and Methods

2.1. Origin of the Bacterial Isolates

Five *Pseudomonas fluorescens* strains (FP3, FP4, FP6, FP7 and FP10) were obtained from Hydrobiology Department, Veterinary Division, National Research Centre, Egypt. These strains were maintained on tryptic soya agar (TSA) with 10 % glycerol and stored at -80°C.

2.2. Preparation of Bacterial Culture Filtrate

From the glycerol stock, fresh cultures were prepared on plates of TSA. A single colony of each bacterial strain was inoculated and grown in a tryptic soya broth (TSB) with constant shaking at 150 rpm for forty-eight hours at 35±2°C. The culture was centrifuged at 6,000 rpm for ten minutes to separate the bacterial cells. The supernatant was centrifuged again at 10,000 rpm for ten minutes at 4°C and passed through a bacteriological filter (0.45 µm). The cell-free supernatant was used to assess its antimicrobial activity against foodborne pathogenic bacteria and mycotoxigenic fungi (Nandakumar *et al*., 2002).

2.3. Extraction of Secondary Metabolites

The cell-free supernatants of *P. fluorescens* strains were extracted three times with equal volumes of ethyl acetate, diethyl ether, butanol, chloroform and hexane at room temperature. Each organic phase was combined and evaporated to dryness at 40°C using a rotary evaporator. The obtained crude extracts were evaluated for their antimicrobial activity.

2.4. Antimicrobial Assay

2.4.1. Test Microorganisms

The antimicrobial activity of the cell-free supernatants of the *P. fluorescens* strains and their fractions were assayed against six species of pathogenic bacteria, two Gram-positive bacteria (*Bacillus cereus* EMCC 1080 and *Staphylococcus aureus* ATCC 13565) and four Gram-negative bacteria (*Salmonella typhi* ATCC 25566, *Escherichia coli* 0157 H7 ATCC 51659, *Pseudomonas aeruginosa* NRRL B-272 and *Klebsiella pneumoniae* LMD 7726). Five fungal species were used for antifungal assay, *Aspergillus flavus* NRRL 3357, *A. parasiticus* SSWT 2999, *A. westerdijikia* CCT 6795, *A. steynii* IBT LKN 23096, and *A. carbonarius* ITAL 204.

2.4.2. Well Diffusion Method

The antimicrobial activity of the cell-free supernatants of *P. fluorescens* strains against the tested bacteria and fungi was evaluated using the well diffusion technique described by Kheiralla *et al*. (2016). A suspension was prepared from each culture of the bacterial and fungal strains and adjusted by turbidimetry to a 0.5 McFarland standard. Using cotton swabs, nutrient agar plates were uniformly inoculated with TSB of bacterial cultures and potato dextrose agar (PDA) medium were inoculated with 50 µL of each fungal culture and uniformly spread using a sterile L-glass rod. A sterilized glass Pasteur pipette was used to make wells of 5 mm in diameter on the agar. Fifty µL of each strain cell-free supernatant were placed individually into each well. Fifty µL of tetracycline (500 µg mL^{-1}) were used as the antibacterial positive control and 50 µL of nystatin (1000 U mL^{-1}) were used as antifungal positive control. The dishes were immediately incubated at 37°C/24 h for bacteria and at 25°C/48 h for fungi. After incubation, the zones of inhibition were measured. The results' average was calculated from at least three replicates for each assay (Emam *et al*., 2018).

2.4.3. Disc Diffusion Method

From the twenty-four-hour incubated nutrient agar slant of each bacterial species, a loop full of the microorganism was inoculated in a tube containing 5 mL of tryptic soy broth. The broth culture was incubated at 35°C for two-six hours until it achieved the turbidity of 0.5 McFarland standard. The activity of the fractions of the *P. fluorescens* strains were examined against all the tested bacterial species using the disc diffusion method of Kirby-Bauer technique (Bauer *et al*., 1966). Using cotton swabs, nutrient agar plates were uniformly inoculated with the tryptic soy broth of the bacterial cultures. A concentration of 10 mg mL^{-1} for each fraction was prepared by dissolving 10 mg in 1 mL of dimethyl sulfoxide (DMSO). Sterilized discs (6 mm) from Whatman No. 1 filter paper were loaded by each fraction and dried completely under sterile conditions. The discs were placed on the seeded plates by using a sterile forceps. The DMSO and tetracycline (500 µg mL^{-1}) represented the negative control and positive control, respectively. Inoculated plates were incubated at 37°C for twenty-four hours, and then the inhibition zones were measured and expressed as the diameter of the inhibition zone including the diameter of the paper disc.

The fungal strains were plated onto Potato Dextrose Agar (PDA) and incubated for five days at 25°C. The spore suspension (2×10^8 CFU mL^{-1}) of each fungus was prepared in a 0.01 % tween 80 solution compared with the 0.5 McFarland standard. Petri dishes containing a PDA medium were inoculated with 50 µL of each fungal culture and uniformly spread using sterile L-glass rod. Sterilized discs (6 mm) were loaded by each fraction (10 mg mL^{-1}) and dried completely under sterile conditions, then were placed on the seeded plates by using a sterile forceps. The DMSO and commercial fungicide nystatin (1000 U mL^{-1}) were considered as a negative and positive control, respectively. The inoculated plates were incubated at 25°C for 48 hours, and then the antifungal activity was assessed by measuring the zone of inhibition (Medeiros *et al*., 2011). The results average was calculated from at least three replicates for each assay.

2.5. GC-MS Analysis

The GC/MS analysis was performed using a Thermo Scientific, Trace GC Ultra / ISQ Single Quadrupole MS, TG-5MS fused silica capillary column (30m, 0.251mm, 0.1 mm film thickness). For the GC/MS detection, an electron ionization system with ionization energy of 70 eV was used; Helium gas was used as the carrier gas at a constant flow rate of 1ml min^{-1}. The injector and MS transfer line temperature was set at 280°C.

The oven temperature was programmed at an initial temperature of 50°C (hold 2 min) to150°C at an increasing

rate of 7°C min^{-1}. then to 270°C at an increasing rate of 5°C min^{-1} (hold 2min) then to 310°C as a final temperature at an increasing rate of 3.5°C min^{-1} (hold 10 min). The quantification of all of the identified components was investigated using a relative peak area percentage. A tentative identification of the compounds was performed based on the comparison of their relative retention time and mass spectra with those of the NIST, WILLY library data of the GC/MS system**.**

2.6. Statistical Analysis

Results were subjected to one-way analysis of variance (ANOVA) of the general liner model (GLM) using SAS (1999) statistical package. The results were the average of three replicates ($p \leq 0.05$).

3. Results and Discussion

3.1. Antimicrobial Activity of P. fluorescens Cell-Free Supernatant

Five strains of *Pseudomonas fluorescens* (FP3, FP4, FP6, FP7 and FP10) were screened for the antimicrobial activity of their cell-free supernatants against six species of foodborne pathogenic bacteria and five species of mycotoxigenic fungi as shown in Table 1. The cell-free supernatants of all five strains showed antibacterial activity against both gram positive and gram negative bacteria. The zone of inhibition ranged from 7.8 to 15.2 mm. the highest antibacterial activity was recorded by the supernatant of FP6 followed by FP3 against *S. aureus* with inhibition zones 15.2 and 15.0 mm, respectively.

The results in Table 2 reveal that the cell-free supernatant of *P. fluorescens* FP10 produced antifungal substances which inhibited the growth of all tested fungi, followed by FP6 and FP7 which inhibited the growth of all tested fungi except *A. flavus* and *A. parasiticus*, respectively. The highest antifungal activity was recorded by FP7 against *A. westrdijikia* with an inhibition zone of 22.0 mm.

The inhibitory activity of *Pseudomonas* spp. against foodborne pathogenic bacteria and fungi has been previously demonstrated. Freedman *et al.* (1989) reported that *P. aeruginosa, P. putida, P. tapaci* and *P. fiuorescens* showed antibacterial activity against *S. typhimurium* and *S. aureus*. Charyulu *et al.* (2009) found that *P. fluorescens* had antibacterial activity against *Salmonella typhi, Streptococcus mutans, Bacillus subtilis,* and *Shigella sonnei*. Also, Rekha *et al.* (2010) screened the antibacterial activity of *P. fluorescens* against ten pathogenic bacteria and the results indicated that *P. fluorescens* had a significant value against *Salmonella typhi, S. sonnei, Streptococcus mutans* and *Bacillus subtilis,* but no activity was observed against *Staphylococcus aureus, Aeromonas hydrophila, V. cholerae, Escherichia coli, K. pneumoniae* and *Serratia marcescens.* Sharma and Kaur (2010) indicated that all of the cultures of the cell-free supernatants from *Pseudomonas* isolates inhibited the growth of *B. subtilis, B. cereus* and *Xanthomonas* sp. as well as human pathogens such as *Salmonella typhi, Shigella* sp. *Klebsiella* sp., *E. coli,* and *Staphylococcus* sp.

Table 1. Antibacterial activity of the cell-free supernatants of *P. fluorescens* strains against some foodborne pathogenic bacteria.

Bacteria	Inhibition zone (mm) (Mean ± S.E)					
	Positive control	FP3	FP4	FP6	FP7	FP10
B. cereus	27.0±1.32^{a}	13.5±1.80^{d}	11.2±1.04^{e}	13.8±1.25^{c}	14.5±0.50^{b}	14.0±2.29^{bc}
S. aureus	26.3±1.04^{a}	15.0±1.80^{bc}	10.5±1.00^{e}	15.2±0.76^{b}	11.5±2.29^{d}	14.8±0.76^{c}
E. coli	26.2±1.04^{a}	14.3±2.93^{b}	12.5±2.29^{d}	11.5±2.29^{e}	13.3±0.76^{c}	12.8±1.25^{c}
S. typhi	24.8±1.61^{a}	10.8±1.60^{e}	12.7±1.89^{c}	11.3±2.08^{d}	13.3±1.04^{b}	12.8±1.25^{bc}
P. aeruginosa	22.5±0.50^{a}	8.0±0.50^{cd}	7.8±0.76^{d}	8.2±0.28^{c}	9.2±0.28^{b}	9.5±1.32^{b}
K. pneumoniae	25.3±1.25^{a}	11.7±2.02^{c}	11.2±1.25^{d}	11.3±2.25^{d}	12.8±1.04^{b}	13.0±2.18^{b}

Means followed by different subscripts within row are significantly different at the 5% level, (n= 3), S.E: standard error, Positive control: tetracycline.

Table 2. Antifungal activity of the cell-free supernatants of *P. fluorescens* strains against some mycotoxigenic fungi.

Fungi	Inhibition zone (mm) (Mean ± S.E)					
	Positive control	FP3	FP4	FP6	FP7	FP10
A. flavus	28.3±0.76^{a}	0	0	0	16.7±0.76^{b}	13.8±2.25^{c}
A. carbonarius	27.2±1.25^{a}	18.3±3.55^{c}	0	14.2±1.04^{e}	21.2±0.76^{b}	15.8±1.60^{d}
A. steynii	29.5±1.32^{a}	10.8±0.76^{d}	0	18.5±0.50^{c}	18.7±3.68b^{c}	19.3±2.92^{b}
A. parasiticus	25.8±1.90^{a}	14.8±2.02^{c}	17.5±1.27^{b}	9.33±0.58^{d}	0	17.8±1.25^{b}
A. westrdijikia	29.2±1.26^{a}	21.2±1.25^{b}	16.0±0.50^{e}	18.2±1.75^{d}	22.0±0.50^{b}	19.2±1.04^{c}

Means followed by different subscripts within row are significantly different at the 5% level, (n= 3), S.E: standard error, Positive control: nystatin.

Moreover, *Pseudomonas* spp. inhibited the growth of *F. oxysporum* as reported by van Peer *et al*. (1990). While, Laine *et al.* (1996) showed that *P. fiuorescens* and *P. chlororaphis* had inhibition effects against *S. typhi, S. aureus, Fusarium oxysporum, F. culmorum* and *A. niger*. Also, *P. fluorescens* had antifungal activity against *P. ultimum* more than *M. phaseolina* and *P. oryzae* (Fuente *et al*., 2004). *P. fluorescens* recorded antifungal activity

against *Fusarium oxysporum* and *F. udum* (Srivastuva and Shalini, 2008). Also, the *Pseudomonas* isolates had antifungal activity against plant pathogens, *i.e. Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., *Rhizoctonia* sp., *Alternaria* sp. and *Trichoderma* sp (Sharma and Kaur 2010). Mushtaq *et al*. (2010) as well as Rajeswari and Kannabiran (2011) reported that the culture filtrate of *P. fiuorescens* had antifungal activity against *Fusarium oxysporum*, *Penicillium italicum* and *Aspergillus niger*.

3.2. Antimicrobial Activity of P. fluorescens Strains Solvent Fractions

The cell-free supernatants of the *P. fluorescens* strains (FP4, FP6, FP7 and FP10) were selected according to their antimicrobial activity and fractionated using different solvents, ethyl acetate, diethyl ether, butanol, chloroform and hexane. The antibacterial activity of different fractions extracted from selected *P. fluorescens* strains are illustrated in Table 3. The chloroform fraction of *P. fluorescens* FP3 had the highest antibacterial activity against *B. cereus*, *E. coli*, *S. typhi* and *K. pneumoniae*, while the butanol fraction showed the highest activity against *S. aureus* and *P. aeruginosa*. On other hand, the chloroform fraction of *P. fluorescens* FP6 and FP7 had antibacterial activity higher than other fractions against all tested bacteria with the exception of *P. aeruginosa*. The highest activity against *P. aeruginosa* was recorded by the hexane fraction of *P. fluorescens* FP6 and the ethyl acetate fraction of *P. fluorescens* FP7 with inhibition zones of 9.3 and 12.7 mm, respectively. Also, the chloroform fraction of *P. fluorescens* FP10 had the highest activity against *B. cereus*, *S. aureus*, *E. coli* and *S. typhi*, while butanol and diethyl ether fractions showed the highest activity against *P. aeruginosa* and *K. pneumoniae*, respectively.

Table 3. Antibacterial activity of the cell-free supernatant fractions of *P. fluorescens* strains

Bacterial extracts	Inhibition zone (mm) (Mean ± S.E)					
	B. cereus	*St. aureus*	*E. coli*	*S. typhi*	*P. aeruginosa*	*K. pneumoniae*
Negative control	0	0	0	0	0	0
Positive control	26.8±0.4^{a}	25.5±1.2^{b}	22.8±1.0^{b}	25.2±0.2^{a}	13.2±0.9^{a}	26.0±1.3^{a}
P. fluorescens FP3						
Ethyl acetate	9.3±1.1^{d}	8.2±0.6^{g}	11.2±0.7^{e}	7.2±0.2^{k}	9.3±0.7^{c}	8.5±1.0^{gh}
Diethyl ether	0	9.3±0.7^{fg}	0	0	0	0
Butanol	9.8±0.6^{d}	13.2±0.2^{e}	8.5±0.3^{g}	7.8±0.2^{k}	11.5±0.6^{ab}	8.3±0.9^{h}
Chloroform	13.3±0.7^{c}	12.0±0.3^{ef}	12.7±1.3^{de}	12.2±0.4^{fg}	8.5±0.8^{c}	10.5±0.9^{fg}
Hexane	0	9.7±0.9^{fg}	8.7±0.7^{g}	10.0±0.3^{hi}	11.8±1.0^{ab}	7.7±0.4^{h}
P. fluorescens FP6						
Ethyl acetate	8.0±0.6^{d}	0	21.5±1.5^{b}	7.8±0.2^{k}	0	8.0±0.6^{h}
Diethyl ether	11.3±0.7^{cd}	13.7±1.6^{de}	8.8±1.0^{fg}	18.3±0.4^{c}	9.0±0.3^{c}	16.2±0.7^{bc}
Butanol	10.8±0.7^{cd}	8.0±0.6^{g}	7.7±0.2^{g}	7.8±0.6^{k}	8.0±0.3^{c}	0
Chloroform	25.8±0.6^{a}	28.7±2.2^{a}	26.8±0.4^{a}	23.3±0.4^{b}	7.8±0.8^{c}	25.0±0.5^{a}
Hexane	8.5±0.3^{d}	9.5±0.3^{fg}	8.2±0.6^{g}	9.3±0.7^{ij}	9.3±0.6^{c}	8.5±0.8^{gh}
P. fluorescens FP7						
Ethyl acetate	19.5±4.3^{b}	13.0±0.8^{e}	11.8±1.5^{b}	13.5±1.2^{e}	12.7±0.2^{ab}	12.7±0.9^{de}
Diethyl ether	11.2±1.0^{cd}	8.0±0.6^{g}	8.5±1.0^{g}	13.3±0.4^{ef}	0	10.5±0.6^{fg}
Butanol	10.8±0.6^{cd}	8.8±0.4^{g}	11.0±0.8^{ef}	10.2±0.4^{hi}	8.0±0.3^{c}	12.7±1.1^{de}
Chloroform	23.5±0.8^{a}	19.8±1.2^{c}	15.5±0.6^{c}	16.3±0.4^{d}	7.8±0.8^{c}	17.5±0.5^{b}
Hexane	11.3±0.4^{cd}	11.7±0.4^{ef}	11.0±0.5^{ef}	11.0±0.3^{gh}	8.5±0.6^{c}	11.3±0.4^{ef}
P. fluorescens FP10						
Ethyl acetate	11.2±2.2^{cd}	11.7±1.3^{ef}	0	10.0±0.3^{hi}	8.2±0.2^{c}	11.5±0.8^{ef}
Diethyl ether	8.2±0.4^{d}	13.2±0.9^{e}	11.2±0.7^{e}	8.2±0.4^{jk}	8.3±0.4^{c}	14.3±0.7^{cd}
Butanol	10.8±0.9^{cd}	11.7±0.6^{ef}	11.0±0.3^{ef}	11.0±0.3^{gh}	12.0±0.9^{ab}	11.0±0.6^{fg}
Chloroform	14.0±0.3^{c}	16.3±1.9^{d}	13.8±0.7^{cd}	13.0±0.6^{ef}	11.2±0.6^{b}	11.7±0.2^{ef}
Hexane	0	0	7.2±0.2^{g}	8.2±0.2^{jk}	0	7.5±0.5^{h}
LSD	3.3	2.7	2.2	1.3	1.7	2.1

Means followed by different subscripts within column are significantly different at the 5 % level (n= 3), S.E: standard error, Negative control: DMSO, Positive control: tetracycline.

The antifungal activity of the cell-free supernatant fractions of *P. fluorescens* strains are illustrated in Table 4. The chloroform fractions of *P. fluorescens* FP6, FP7 and FP10 showed the highest antifungal activity against all tested mycotoxigenic fungi, while the chloroform fraction of *P. fluorescens* FP3 had the highest activity against *A. flavus*, *A. carbonarus* and *A. parasiticus* since ethyl acetate and butanol fractions showed highest inhibition zones against *A. niger* and *A. westerdijikia*, respectively. The highest antifungal activity was observed by the chloroform fraction of *P. fluorescens* FP6 against *A. niger* with a 24.0 mm inhibition zone.

Generally, *P. fluorescens* FP6, FP7 and FP10 chloroform fractions showed highest antimicrobial activity against the tested foodborne pathogenic bacteria and mycotoxigenic fungi. So, the bioactive compounds

in these fractions should be identified using Gas Chromatography Mass Spectroscopy (GC/MS).

Fassouane *et al*. (1995) found that the extraction of *P. fluorescens* FSJ-3 cell free supernatant with n-butanol exhibited excellent antimicrobial activity against several species of bacteria, yeast and fungi, including *P. aeruginosa*, *Staphylococcus aureus*, *Candida albicans*, *Aspergillus niger* and *A. fumigatus*. The crude extracts of *P. fluorescens* isolates inhibited the growth of *Dreschelaria oryzae*, *R. solani*, *Magnaporthe grisea* and *Sarocladium oryzae* completely (Reddy *et al*., 2007). In a previous study, the antifungal activity of ethyl acetate and methanol extracts of *P. fluorescens* Pf3 showed that they completely inhibited the mycelial growth of *Botrytis fabae* compared with the hexane extract. Meanwhile, the antifungal activity of the hexane extract of *P. fluorescens* Pf8 showed complete inhibition of the *B. fabae* growth compared with the ethyl acetate and the methanol extracts (Alemu and Alemu, 2013).

Table 4. Antifungal activity of cell-free supernatant fractions of *P. fluorescens* strains**.**

Bacterial extracts	Inhibition zone (mm) (Mean ± S.E)				
	A. flavus	*A. carbonarus*	*A. niger*	*A. parasiticus*	*A. westerdijikia*
Negative control	0	0	0	0	0
Positive control	15.8±0.4^{a}	22.7±0.7^{a}	15.3±0.4^{c}	15.5±0.3^{c}	12.3±0.6^{d}
P. fluorescens FP3					
Ethyl acetate	8.5±0.8^{de}	7.8±0.6^{ef}	9.8±0.6^{d}	0	0
Diethyl ether	0	7.3±0.2^{f}	0	0	0
Butanol	9.0±0.6^{de}	8.5±0.3^{ef}	8.8±0.2^{de}	9.0±0.8^{d}	9.2±0.4^{ef}
Chloroform	9.2±0.2^{d}	9.5±1.3^{de}	0	9.3±1.1^{d}	9.0±0.6^{ef}
Hexane	0	8.3±0.7^{ef}	0	8.2±0.4^{d}	8.8±0.2^{f}
P. fluorescens FP6					
Ethyl acetate	9.2±1.3^{d}	9.0±0.3^{ef}	8.0±0.6^{e}	8.8±1.0^{d}	0
Diethyl ether	9.0±1.3^{de}	8.8±0.4^{ef}	9.3±0.2^{de}	9.7±1.0^{d}	7.8±0.4^{f}
Butanol	9.0±0.8^{de}	0	0	9.5±1.3^{d}	0
Chloroform	20.0±0.3^{a}	22.2±1.1^{a}	24.0±1.3^{a}	20.7±1.5^{a}	22.0±1.0^{a}
Hexane	7.3±0.3^{e}	9.0±1.2^{ef}	9.7±0.4^{d}	0	11.2±0.6^{de}
P. fluorescens FP7					
Ethyl acetate	11.±0.4^{c}	11.7±0.4^{c}	8.0±0.6^{e}	8.5±1.0^{d}	8.7±0.6^{f}
Diethyl ether	7.8±0.6^{de}	0	0	8.3±0.7^{d}	0
Butanol	0	11.2±0.6^{cd}	9.2±1.0^{de}	7.8±0.6^{d}	8.0±0.6^{f}
Chloroform	18.8±1.2^{a}	23.8±1.2^{a}	19.3±1.0^{b}	18.2±1.9^{b}	19.5±2.4^{b}
Hexane	8.3±0.3^{de}	9.5±0.3^{de}	0	8.2±0.4^{d}	9.3±0.6^{ef}
P. fluorescens FP10					
Ethyl acetate	11.3±0.9^{c}	0	8.5±0.3^{de}	0	0
Diethyl ether	7.5±0.3^{de}	9.0±0.3^{ef}	8.7±0.4^{de}	8.0±0.6^{d}	0
Butanol	0	9.5±1.6^{de}	8.5±0.3^{de}	8.7±0.9^{d}	0
Chloroform	11.7±0.2^{c}	14.7±0.7^{b}	9.3±0.6^{de}	9.5±0.3^{d}	15.0±2.0^{c}
Hexane	0	0	0	0	0
LSD	1.7	2.0	1.5	2.3	2.2

Means followed by different subscripts within column are significantly different at the 5 % level (n= 3), S.E: standard error, Negative control: DMSO, Positive control: nystatin.

3.3. Identification of the Compounds Using GC/MS

The results pertaining to the GC/MS analysis of *P. fluorescens* FP6, FP7 and FP10 chloroform fractions are illustrated in Figure 1 and Table 5. They reveal the presence of eight metabolites in the chloroform fraction of *P. fluorescens* FP6 with a retention time ranging from 11.32 to 35.46 minutes. The maximum peak area was identified as Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(propylmethyl)- with an area percentage of 41.30 %, followed by Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- ($C_{14}H_{16}N_2O_2$) as 21.60 % and (2S,6R)-2,6-Dibutyl-4-methylpiperdine ($C_{14}H_{29}N$) as 12.90 %. Meanwhile, the minimum peak was identified as Methaqualone ($C_{16}H_{14}N_2O$) and the peak area percentage was 0.98 %.

On the other hand, five compounds were identified from the chloroform fractions of *P. fluorescens* FP7, as 4-Bromo-5-methyl-1-(phenylmethyl) imidazole ($C_{11}H_{11}BrN_2$), 3-Ethoxy-4-methoxyphenol ($C_9H_{12}O_3$), Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)-, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(propyl

methyl)- and Cyclotrisiloxane, hexamethyl- ($C_6H_{18}O_3Si_3$) with peak area percentages of 0.76, 5.68, 25.35, 51.60 and 16.61%, respectively. Regarding to the chloroform fraction of *P. fluorescens* FP10, four compounds were found as 3-Ethoxy-4-methoxyphenol, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-, Pyrrolo[1,2-a] pyrazine-1,4-dione,hexahydro-3-(propylmethyl)-and Cyclo trisiloxane, hexamethyl- with peak area percentages of 4.14, 26.32, 47.40 and 22.14 %, respectively. Two Pyrrolo compounds were found in all of the fractions as the main compounds, Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(propylmethyl)- and Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-. Also, Cyclotrisiloxane, hexamethyl- and 3-Ethoxy-4-methoxyphenol were found in all *P. fluorescens* chloroform fractions.

The antibacterial metabolite of *P. fluorescens* strains showed activity against both Gram-negative and Gram-positive bacteria. Also, they have broad-spectrum antifungal activty against phytopathogenic and toxigenic fungi. Narasaiah (2016) reported that *Streptomyces albus* CN-4 secondary metabolites which contain Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- had antibacterial activity against *Bacillus megaterium, B. subtilis, E. coli, Pseudomonas aeruginosa, Staphylococcus aureus* and *Xanthomonas campestris*. Also, they had antifungal activity against *Aspergillus niger, Candida albicans* and *Fusarium solani*. The pure Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methyl propyl)- had antibacterial activity against pathogenic bacteria that cause serious fish and human diseases (Pandey *et al*., 2010). Dash *et al*. (2009) found that Pyrrolo [1,2-a] pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)- isolated from a sponge-associated marine bacteria prevented biofilm formation by *Loktanella honkongensis* and *Vibrio halioticoli*. Also, Pyrrolo [1,2-a] pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)- isolated from marine sponge *Spongia officinalis* exhibited potent antibacterial and antifungal activity (Sathiyanarayanan *et al*., 2014). Melo *et al*. (2014) indicated that the chloroform extract of Antarctic endophytic fungus exhibited strong antibacterial activity, mainly against *P. aeruginosa*, *Enterococcus faecalis* and *E. coli*. They added that, Pyrrolo[1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- and Pyrrolo[1,2-a]pyrazine-1,4-dione,hexahydro-3-(phenyl-methyl)- from the fungus extract had potential antimicrobial compounds. Also, pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-3-(2-methyl propyl)- purified from *Streptomyces* sp. had antibacterial activity against *E. coli*, , *Proteus vulgaris, P. aeruginosa, S. paratyphi, Enterobacter* spp. and *S. aureus, B. cereus* (Manimaran *et al*., 2017).

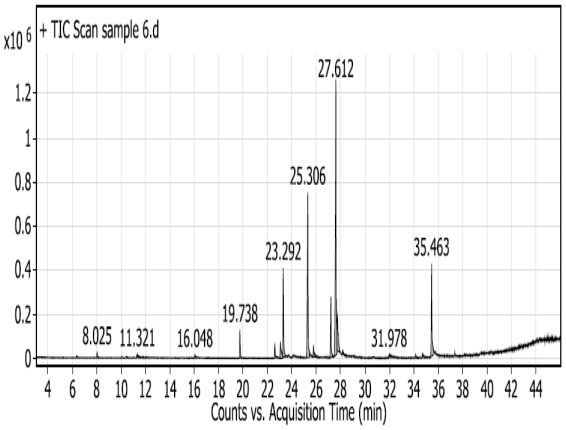

Figure 1. GC-MS chromatogram of *P. fluorescens* FP6 chloroform fractions.

Table 5. GC-MS analysis of chloroform fractions *of P. fluorescens strains* (FP6, FP7 and FP10).

No	RT	Chemical	Structure	Chloroform fractions (Peak area %)		
				Pf 6	Pf 7	Pf 10
1	11.32	4-Bromo-5-methyl-1-(phenylmethyl)imidazole	$C_{11}H_{11}BrN_2$	1.18	0.76	--
2	16.05	Methaqualone	$C_{16}H_{14}N_2O$	0.98	--	--
3	19.70	beta-Lonipinene	$C_{15}H_{24}$	6.13	--	--
4	23.01	3-Ethoxy-4-methoxyphenol	$C_9H_{12}O_3$	3.11	5.68	4.14
5	23.30	(2S,6R)-2,6-Dibutyl-4-methylpiperdine	$C_{14}H_{29}N_4$	12.90	--	--
6	25.30	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	$C_{11}H_{18}N_2O_2$	21.60	25.35	26.32
7	27.61	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(propylmethyl)-	$C_{14}H_{16}N_2O_2$	41.30	51.60	47.40
8	35.46	Cyclotrisiloxane, hexamethyl-	$C_6H_{18}O_3Si_3$	12.80	16.61	22.14

4. Conclusion

The present *in-vitro* study reveals that all of the studied cell-free supernatants of the *P. fluorescens* strains (FP3, FP4, FP6, FP7 and FP10) were more effective as antibacterial against all the tested pathogenic bacteria. Meanwhile, only the *P. fluorescens* FP10 strain was more effective as antifungal against all studied mycotoxigenic fungi. The main compound identified in chloroform fractions were Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(propylmethyl)- and Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)- that have been proved to possess a biological activity against pathogenic bacteria and toxigenic fungi. Finally, data in the current study reveal that cell-free supernatants and chloroform fractions of *P. fluorescens* strains displayed potential antimicrobial activity against foodborne microorganisms that could be applied as food biopreservative agents.

Conflict of Interest

The authors have no conflicts of interest to declare.

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