Antibacterial Activity of Ethyl Acetate Extract of Endophytic Fungus (*Paraconiothyrium Brasiliense*) Through Targeting Pathogenesis Related Dihydropteroate Synthase (DHPS)

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Abstract

Endophytic fungi are innumerable sources of bioactive metabolites with various biomedical applications. Hence, this study was aimed to isolate and identify the endophytic fungus *Paraconiothyrium brasiliense* (PB) from the fruit of *Capsicum annuum* and evaluated their biochemical, antimicrobial, antioxidant, cell viability, and cytotoxicity behavior. Results showed that ethyl acetate extract (EAE) of PE contains flavonoid (31.53 ± 0.9 mg of QE/g of extract) and phenolic (2.59 ± 0.06 mg of GAE/g of extract). The PB-EAE showed significant antioxidant activity in terms of free radicals such as DPPH, ABTS, and Fe^{3+} scavenging. The antioxidant potential of PB-EAE reflects in normal cell viability and anticancer activity against prostate cancer (PC3) cells. The bacterial inhibitory activity of PB-EAE was equal to commercial antibiotic tetracycline hydrochloride (TCH). Further, GC-MS analysis confirmed the presence of volatile molecules such as o-cymene ($\text{CH}_3\text{C}_6\text{H}_4\text{CH}(\text{CH}_3)_2$), dipentene ($\text{C}_{10}\text{H}_{16}$), γ -terpinene ($\text{C}_{10}\text{H}_{16}$), 4-carvomenthenol ($\text{C}_{10}\text{H}_{18}\text{O}$), heneicosane ($\text{CH}_3(\text{CH}_2)_{19}\text{CH}_3$), 2,5-piperazinedione ($\text{C}_4\text{H}_6\text{N}_2\text{O}_2$). Also, molecular docking analysis demonstrated the significant interactions between the compounds of PB-EAE and Dihydropteroate Synthase (Protein ID:5JQ9; DHPS). This study revealed that endophytic *P. brasiliense* from *Capsicum annuum* could be a novel source for the isolation of antimicrobials.

Introduction

Emerging microbial infections, diseases, and antimicrobial resistance (AMR) by bacteria, fungi, and parasites are believed to be the potential risks to living beings [1]. Hence, there is the need to discover and develop efficient drugs against microbial infection-associated diseases. At the same time, plant endophytic fungi are widely reported as the sources of novel secondary metabolites such as terpenes, terpenoids, polyketides, alkaloids, and flavonoids [2, 3]. Those obtainable secondary metabolites from endophytes have significant biological properties such as antibacterial, antifungal, antiviral, antioxidant, anti-inflammatory, anticancer, antidiabetic, insecticidal, and immunomodulatory [4]. Endophytic fungi are symbiotic microorganisms that are colonized in the living tissues of the plants and other living materials and assist to defend the various biological, environmental factors [5, 6]. Also, endophytic fungi colonization in plants plays a key role in determining secondary metabolites production [7]. Endophytic fungi were often isolated from various sources (terrestrial and aquatic plants, marine organisms (algae, seagrasses, vertebrates, and invertebrates) [8–11]. The group of bioactive secondary metabolites and their biological activities differed according to the sources of isolation [12, 13].

The bioactive molecules such as paclitaxel, resveratrol, camptothecin, huperzine A, and podophyllotoxin are known to isolate from endophytic fungi *Penicillium* sp., *Aspergillus* sp., *Alternaria* sp., *Trichoderma* sp., *Fusarium* sp., and *Phomopsis* sp., [14]. Among the endophytic fungi, *P. brasiliense* strains are explored from different plant sources such as Chinese maple leaves [15], *Chloranthus japonicus* [16]. The isolation of novel *Paraconiothyrium* sp., such as *P. africanum*, and *P. brasiliense* reported from *Prunus persica*, *P. hawaiiense* from *Sophora chrysophylla*, and *P. variabile* from *Actinidia chinensis* [17]. A study reported that bisbolane sesquiterpenoids isolated from endophytic fungus *P. brasiliense* (M3-3341) associated with the *Acer truncatum* [18]. Further, bisbolane sesquiterpenoids (brailamide E (1)) and

derivative compounds showed significant anticancer activity when testing with the MCF-7 cell line [18, 19]. A study reports that brasilamide K-N (1–4) and new bergamotane sesquiterpenoids were isolated from the *P. brasiliense* through the fermentation process [20]. Interestingly, paraconifuranones A-H and I-M were reported from *Acrida cinerea* gut-associated *P. brasiliense* [21, 22]. A new ketol compound (sporulosol) was isolated from *P. sporulosum* and found that showed significant anticancer activity against different cancer cell lines [23]. The recent study reports that ten new sesquiterpenoids such as paraconiothins A-J were isolated from *P. brasiliense* ECN258 associated with *Cinnamomum camphora* [24]. Dihydropteroate synthase (DHPS) is a key enzyme involved in dihydrofolate synthesis in microorganisms and plants. Further, dihydrofolate is required to make the nitrogenous base (purines and pyrimidines) of DNA and RNA. Hence the inhibition of the DHPS enzyme is an important target for achieving antimicrobial activities [25].

Therefore, endophytic fungi could be the key source for the drug discovery and development of several diseases. However, among the large group of endophytic fungi, a limited number of fungal secondary metabolites were reported. Further, isolated novel compounds and their derivatives are not sufficiently studied for their biological functions. To the overview of the *Paraconiothyrium* sp., were reported several new terpenoid derivatives but those compounds are just tested their cytotoxicity and not studied their other biological functions such as antioxidant, antibacterial, antidiabetic, etc. Hence, this study aimed to evaluate biochemical and biological activities of ethyl acetate extract of endophytic fungus *P. brasiliense* from *Capsicum annum*. The biochemical studies include the analysis of total phenol and flavonoid content and volatile compounds analysis by gas chromatography-mass spectrum (GC-MS) analysis. The biological studies include antioxidant, antibacterial, cytotoxic activities. Also, volatile molecules interaction was tested with DHPS enzyme through molecular docking analysis.s

Materials And Methods

Sample collection and Isolation of endophytic fungus

The healthy bell pepper (*Capsicum annum* L.) was purchased from the local former market in Chuncheon, Republic of Korea in May 2020. Then the bell pepper samples were brought to the laboratory and impurities were removed by washing with tap water and distilled water. For the surface sterilization, the bell pepper samples were cut as pieces (approximately 5 × 5 mm) washed with ethanol (95%), sodium hypochlorite (4% chlorine), and ethanol (95 %) for 1 min, 4 min, and 30 s, respectively. Followed by the bacterial cell suspension was prepared by putting the 5 pieces of bell pepper in 10 mL of 0.9 % sterile saline solution and vortexed for 2 min. Then, 50 µL of ten-fold diluted cell suspension was inoculated on potato dextrose agar (PDA) supplemented with tetracycline hydrochloride (20 µg/mL) and incubated at 26 ± 2°C for 72 h. Afterward, about 10 unique single fungal colonies were subculture on PDA plates. Finally, the unique fungal strain (KNUFCPF01) was selected for further molecular identification based on morphological characteristics and antibacterial activities.

Molecular identification

Fungal strain (KNUFCPF01) was cultured on PDA plates for 3–5 days. Approximately, 100 mg of fungal mycelia were collected without agar and used for gDNA extraction according to the CTAB method reported elsewhere. The isolated fungal gDNA was used as a template to amplify the marker gene internal transcribed spacer (ITS) using the primers ITS1 and ITS2 [26]. The PCR product separated using the 1% agarose gel electrophoresis and the fragment were isolated and sequenced at macrogen (<https://www.macrogen.com/ko/main/index.php>). Afterward, the ITS gene sequences data were compared with available ITS data in the NCBI database (<https://www.ncbi.nlm.nih.gov/>) through Nucleotide BLAST search. The phylogenetic tree was formed to demonstrate the similarity between the existing ITS gene sequence of the same strain along with the out-group of *Rhizopus oryzae* were obtained from the NCBI GenBank database by the neighbor-joining method using MEGA X software.

Extraction of metabolites

The fungal strain KNUFCPF01 was cultured in the potato dextrose broth (PDB) at $26 \pm 2^\circ\text{C}$ under static conditions for 14 days. Afterward, the culture was filtered using Whatman No 1 filter paper. The obtained culture filtrate was mixed with ethyl acetate 5:1 ratio and collected the upper organic layer. Further, the extraction process was repeated 3–4 times and all the extracts pooled together and condensed using a rotary evaporator at 45°C . Finally, the ethyl acetate extract (EAE) was collected and stored at room temperature.

Total phenol and flavonoid content

The total phenol and flavonoid content in PB-EAE were evaluated according to earlier reports [27]. In brief, total phenol content was determined by mixing Folin-phenol reagent (200 μL), 100 μL EAE (1 mg/mL), and saturated sodium bicarbonate (200 μL). After 10 min incubation at room temperature, 100 μL of aliquots were read at 760 nm using a UV-visible spectrophotometer. For the total flavonoid content determination, 100 μL of EAE mixed with ethanol (150 μL), distilled water (280 μL), 10% of aluminum chloride (10 μL), and 1 M potassium acetate (10 μL). Further, 100 μL of reaction mixture read at 415 nm using a spectrophotometer. The total phenol and flavonoid content were calculated as gallic acid (GAE) and quercetin (QE) equivalent, respectively.

GC-MS analysis

The existence of volatile compounds in PB-EAE was determined using Gas chromatograph/mass selective detector (GC/MSD, Agilent Technologies, USA, 7890A, 5975C) analysis. About 10 mg of EAE was dissolved in 10 mL of methanol. Then 2 μL of sample loaded into the Agilent column (DB-5 ms (30 $\times$ 0.25 mm, 0.25 μm), column flow at 1 mL/min with helium gas (He), and interface temperature at 280°C . The volatile compound was detected by the mass selective detector with the scan range of 40–400 m/z and matched with the electronic library (W8N05ST.L).

Antioxidant and enzyme inhibition assay

Free radical scavenging ability of different concentrations of PB-EAE was determined through DPPH, Ferric reducing and ABTS radical scavenging assay according to the earlier reports [27]. The enzyme (α -amylase and α -glucosidase) inhibitory activities of EAE were determined according to the earlier report [28].

Cell culture assay

The human embryonic kidney cell line (HEK-293 cells), and prostate cancer cell line (PC3 cells) were obtained from the Korea cell line bank. The cells were cultured in a T-25 culture flask using the high glucose DMEM for HEK-293 cells and RPMI media for PC3 cells supplemented with antibiotic penicillin and streptomycin, respectively. Both cells were maintained in a 5 % CO₂ incubator at 37°C. Cell viability of PB-EAE was determined using a WST viability assay kit. Both cells (1×10^4) were seeded into the 96-well plate individually containing the respective cell culture media and incubated for 24 h. Subsequently, 10 μ L/well of different concentrations of PB-EAE were treated and incubated for 12 h. Then, WST reagent (10 μ L/well) was added and continued the incubation for 1 h. Afterward, the 96-well culture plate was read at 450 nm. The cell viability (%) was determined according to the earlier report [29]. Further, the IC₅₀ of the EAE for PC3 cells was evaluated using different apoptotic fluorescent staining assays according to the earlier report [30].

Antibacterial activity

The bacterial inhibition ability of PB-EAE was evaluated by well diffusion assay in both Gram-positive (*Bacillus cereus* (ATCC 14579), *Staphylococcus aureus* (ATCC 19095), and *Listeria monocytogens* (ATCC 19115)) and Gram-negative (*Escherichia coli* (ATCC 43888) and *Salmonella enterica* (ATCC 14028)) bacterial pathogens. The bacterial pathogens were obtained from the American Type Culture Collection (ATCC), University Blvd, Manassas, US. For the antibacterial assay, each bacterial strain was cultured in nutrient broth at the density of 1×10^9 (colony-forming units (CFU)/mL) and it was swapped onto a Muller-Hinton agar (MHA) plate using a cotton swab. Then, wells were made by metal cork-borer, the different concentrations of EAE were added to each well along with the positive control (tetracycline hydrochloride). Afterward, these plates were incubated at 37°C overnight. After incubation, the antibacterial activity was determined in terms of the zone of inhibition. The minimum inhibitory concentration of EAE determined by the broth dilution method was reported elsewhere [31]. In brief, different concentration of 10 μ L of EAE of endophytic fungus (1000, 500, 250, 125, 62.5, 31.25, 15.62, 7.8, 3.9 μ g/mL) was treated with each bacterial culture (5×10^5 CFU/mL) in 100 μ L of MHB medium in 96 well plate for 24 h. After incubation, the plates have measured the absorbance at 600 nm for calculation of MIC.

In vivo molecular docking analysis

Further, the antibacterial molecular mechanism of volatile molecules in the PB-EAE was demonstrated by molecular docking analysis with *Yersinia Pestis* dihydropteroate Synthase (DHPS) enzyme (5JQ9). Firstly, the volatile molecules of EAE were tested and selected depends on their drug-likeness giving to Lipinski's rules [32]. The volatile molecule structure was developed using colonial smiles from NIH PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) and energy minimalized by UCSF chimera 1.14. Besides, the target bacterial DHPS enzyme (PDB ID: 5JQ9) was obtained from the RCSB protein data bank (<https://www.rcsb.org/>). The molecular docking analysis was done in Argus Lab (Version 4.0.1), and then the results of interactions were visualized using BIOVIA Discovery Studio Visualizer (v20.1.0.19295).

Statistical analysis

All the experiments were done individually at least thrice, and results were shown as the means with standard deviation (SD). The statistical significance was determined using one-way analysis variance (ANOVA). $p < 0.05$, statistically considered as the significance.

Results And Discussion

Isolation and molecular identification of strain KNUFCPF01

Among the different fungal isolates, the endophytic fungal strain KNUFCPF01 (Fig. 1A) was selected and identified by sequencing of the ITS region (ITS1, 5.8S, and ITS2 of gDNA. The gene sequencing of strain KNUFCPF01 was identified as *P. brasiliense* (MT269522). A study reported that endophytic fungus *Paraconiothyrium* sp. was isolated from the root of *Capsicum annum* [33]. Those finding was supported the existence of *Paraconiothyrium* sp. in *Capsicum annum*. Further, the phylogenetic relationship among the *P. brasiliense* strains and other genera was studied and shown in Fig. 1B. The neighbor-joining method of MT269522, a phylogenetic tree constructed using gene sequences of partial ITS1 and ITS2, complete 5.8S rRNA in MEGA X. The phylogenetic tree showed that strain MT269522 was clustered the similarity with *Paraconiothyrium* strains whereas *R. oryzae* was used as an outgroup.

Biochemical analysis

The TPC and TFC of PB-EAE showed 2.59 ± 0.06 mg/g of gallic acid equivalent and 31.53 ± 0.90 mg/g of quercetin equivalent (Table 1). The results indicated that the TPC of PB-EAE was lower than TFC. Similarly, EA extract of endophytic fungal strain *Fritillaria unibracteata* var. exhibits the different concentrations of phenolic, flavonoid saponin that responsible for the biological properties [34]. The phenolics, flavonoids, and terpenoids are widely reported as antioxidant, antibacterial, and anticancer compounds [34–36]. Further, the volatile compound profile of PB-EAE was determined by GC-MS analysis (Fig. 2). Table 2 shows the retention time, peak area, compound name, molecular formula, and molecular weight of the volatile compound from EAE of *P. brasiliense*. The results predicted that existence of 12 major bioactive volatile compounds such as 2,3-butylene glycol ($\text{CH}_3\text{CH}(\text{OH})\text{CH}(\text{OH})\text{CH}_3$), isopentyl acetate ($\text{CH}_3\text{COOCH}_2\text{CH}_2\text{CH}(\text{CH}_3)_2$), o-cymene ($\text{CH}_3\text{C}_6\text{H}_4\text{CH}(\text{CH}_3)_2$), dipentene ($\text{C}_{10}\text{H}_{16}$), γ -terpinene

(C₁₀H₁₆), 3,5-nonadien-7-yn-2-ol (C₉H₁₂O), 2-phenylethanol (C₆H₅CH₂CH₂OH), 4-carvomenthenol (C₁₀H₁₈O), 2-phenethyl acetate (CH₃COOCH₂CH₂C₆H₅), heneicosane (CH₃(CH₂)₁₉CH₃), 2,4-di-tert-butylphenol ([C(CH₃)₃]₂C₆H₃OH), and 2,5-piperazinedione (C₄H₆N₂O₂). The 2,3-butylene glycol was reported a substantial presence in the seeds of *Dimocarpus longan* Lour. which is used for wine production and facilitates antioxidant properties [37]. 2, 3-butylene glycol helpful for plant-microbe interactions and act as an elicitor [38]. Also, the derivative of 1, 3-butylene glycol exhibited potent antibacterial and antifungal activities [39]. Similarly, the compound isopentyl acetate from endophytic *Bacillus subtilis* inhibits the growth and sporulation of *Curvularia lunata* [40]. Cymene isomers are reported to possess insecticidal and repellent properties [41]. Also, the recent studies confirmed that o-cymene has an inhibitory effect against the H1N1 influenza virus [42] and coronavirus 2 [43]. The study assumes that the maximum existence same molecular weight derivative of dipentene, and γ-terpinene in black pepper was the reason for antioxidant activity [44]. The 4-carvomenthenol is a fragrance molecule [45] and it has the property to recover from allergic rhinitis and asthma syndrome [46]. 2,5-Piperazinedione derivatives from *Penicillium griseofulvum* exhibited immunosuppressive activity in anti-CD3/anti-CS28 stimulated murine splenocytes [47]. Also, diketopiperazines derivatives were isolated from endophytic fungus *Bionectria* sp. Y1085 showed significant antibacterial activity [48]. Further, ascotoxin reported from EA extract of *Capsicum annum* root endophytic fungus *Paraconiothyrium* sp. inhibit the seed germination of wild grass (*Echinochloa crus-galli*) [33]. Also, the study reported that 1,8-dihydroxyanthraquinone from endophytic *Paraconiothyrium* sp. from *Zingiber officinale* Rosc. and it was shown in broad spectrum of antimicrobial activity against human pathogenic bacteria and phytopathogenic fungi [49].

Antioxidant activity

Antioxidant molecules are functioning as the substrate to quenching the incomplete oxygen reduction of reactive oxygen species (ROS) formation in mitochondria thereby protects the cells from oxidative damage [50, 51]. Also, the increased level of ROS oncogenic signaling molecules initiates uncontrolled cell proliferation [51]. To reduce chronic and degenerative diseases, antioxidant molecules have been used widely from different sources (bacteria, algae, fungi, and plants) [52, 53]. The present study showed that EAE of *P. brasiliense* contains a wide range of antioxidant molecules which are identified from biochemicals content and volatile compounds profile analysis. Hence, EAE of *P. brasiliense* was tested for inhibition of ROS formation by DPPH and ABTS radical scavenging assay and ferric reducing power assay (Table 1). According to the experimental result, the EAE of *P. brasiliense* showed the IC₅₀ concentration for DPPH radical scavenging at 383.51 ± 6.97 µg/mL compared to ascorbic acid at 69.11 ± 0.99 µg/mL. Similarly, a previous study reported that EA extract of *Paraconiothyrium* sp. P83F4/1 showed potential DPPH activity [54]. Also, the EA extract of *P. brasiliense* showed the IC₅₀ concentration for ABTS radical scavenging at 29.57 ± 1.56 µg/mL compared to ascorbic acid 41.84 ± 1.37 µg/mL. The same way EA extract of endophytic fungus *Diaporther* sp. showed ABTS radical scavenging activity [55]. In addition, EA extract of *P. brasiliense* showed the IC₅₀ concentration for ferric reducing power at 358 ±

10.33 µg/mL compared to ascorbic acid 317.6 ± 4.11 µg/mL. Similarly, endophytic fungal (*Fritillaria unibracteata* var.) extract reported that required a higher concentration for reduction of ferric ion to ferrous [34]. Based on the different antioxidant assays, we concluded that EA extract of *P. brasiliense* could be the potential source for the isolation of antioxidant molecules.

Cell viability

Cell viability assay is used to determine how the metabolites or drug molecules affect cell proliferation and its biochemical changes. In order to test the cell viability effect, EAE of *P. brasiliense*, HEK-293 cells, and PC3 cells were used, and cell viability was determined by the WST assay method [56]. The EAE of *P. brasiliense* treated PC3 cells were showed the IC₅₀ concentration at 187.3 ± 9.29 µg/mL while HEK293 cells did not show any IC₅₀ concentration up to a tested concentration of 1000 µg/mL (Fig. 3). Similarly, EA extract of endophytic *Phomopsis* sp. did not affect the normal cell (HeLa cells) proliferation while showed the anticancer activity against breast cancer cell line (MCF-7) [57]. Hence, the results revealed that EAE of *P. brasiliense* showed toxicity against cancer cells but not in a normal cell. Further, EAE of *P. brasiliense* treated PC3 cell viability and apoptotic changes evaluated using different fluorescent staining. The results of nuclear staining (PI and DAPI) demonstrated that more dead cells population in EAE of *P. brasiliense* treatment compared to untreated control PC3 cells (Fig. 4). The mitochondrial membrane potential of cells was determined using a rhodamine 123 fluorescent stain. The results indicated that EAE of *P. brasiliense* treated cells were lost the mitochondrial membrane potential compared to control. Also, the higher ROS elevation was observed in EAE of *P. brasiliense* treated cells but not observed in control cells evaluated by DCFH-DA staining. The overall results revealed that EAE of *P. brasiliense* had the excellent cytotoxic behavior on PC3 cells but less toxic to normal cells. Similarly, the study reported that brasilamide E (1) from *P. brasiliense* showed the toxicity to cancer cells (HO8910, MCF-7, and HeLa) while non-toxic to normal cell (NIH3T3)[19]. Also, ketal compound sporulosol exhibited considerable cytotoxicity to HeLa, T24, A549, HCT116, and SH-SY5Y cells [23]. But isolated eight furanone compounds (paraconfuranones A-H) from *P. brasiliense* MZ-1 did not show any toxicity to A549, HepG2, and CaSki cells [22].

Antidiabetic assays

The digestive enzymes play a major role in converting complex sugar molecules into simple sugar molecules through enzymatic digestion [58]. Even if in the case of diabetic patients, blood sugar might be increased than normal people due to the enzymatic action. Hence, the inhibition of enzyme function subsequently decreased the blood sugar level. In the present study, *P. brasiliense* EA extract did not show considerable digestive enzyme inhibitory activity against α-amylase and α-glucosidase (Data not shown).

Antibacterial activity

Antibacterial agents are used to killing the bacteria (bactericidal) or slow down the bacterial growth (bacteriostatic). Most of the antibacterial agents were initially isolated from natural sources and then they have been synthesized their own or derivative form for the applications [59, 60]. However, prolonged use of drugs creates antibiotic-resistant bacterial strains which urge the discovery of novel antibiotics. Hence, EAE of *P. brasiliense* was evaluated for its antibacterial efficacy with bacterial pathogens such as *B. cereus*, *S. aureus*, *L. monocytogenes*, *E. coli*, and *S. enterica* (Fig. 5). The results showed that EAE of *P. brasiliense* had substantial inhibitory activity against both Gram-positive and Gram-negative bacteria. The inhibitory activity depending on the concentration that means when increasing/decreasing the EA extract concentration, the bacterial inhibition activity was increased/decreased, respectively. Among the different concentrations of EAE of *P. brasiliense*, 50 μ L of maximum tested concentration (1000 μ g/mL) showed the zone of inhibition at 17 ± 1.2 mm for *B. cereus*, 22 ± 1.4 mm for *S. aureus*, 24 ± 1.4 mm for *L. monocytogenes*, 8 ± 0.4 mm for *E. coli*, and 21 ± 1.6 mm for *S. enterica* (Table 3). Also, the positive control 20 μ L TCH (1000 μ g/mL) exhibited the zone of inhibition 21 ± 1.2 mm for *B. cereus*, 22 ± 1.2 mm for *S. aureus*, 23 ± 1.4 mm for *L. monocytogenes*, 12 ± 1.2 mm for *E. coli*, and 21 ± 1.4 mm for *S. enterica*. Interestingly, the EAE of *P. brasiliense* showed significant antibacterial activity ($p < 0.05$), which is the same as the antibiotic TCH. The minimum inhibitory concentration of EAE of *P. brasiliense* showed *B. cereus* at 31.25 μ g/mL, for *S. aureus* at 15.62 μ g/mL, *L. monocytogenes* at 15.62 μ g/mL, *E. coli* at 62.5 μ g/mL, and *S. enterica* at 15.62 μ g/mL. Similarly, endophytic *Paraconiothyrium* sp. isolated from *Zingiber officinale* Rosc. showed considerable antibacterial activity against clinical pathogens [49]. But the antibacterial properties of *Paraconiothyrium* sp. were studied inadequately the research gap could be filled by future studies.

Molecular docking analysis

Molecular docking analysis was performed to identify the interaction of metabolites with the molecular target of anti-bacterial activity. The inhibition of dihydropteroate synthase (DHPS) is one of the key mechanisms to kill the bacterial or inhibit bacterial growth through pterin-sulfonamide conjugation [61]. According to those strategies, the identified volatile compounds from EA extract of *P. brasiliense* interact with DHPS. The results indicated that out of 12 volatile compounds, 10 compounds completely satisfied Lipinski's rules (Table 4). Further, molecular docking analysis of major volatile compounds was tested with DHPS (5JQ9) was shown in Table 5 and Fig. 6. The docking results showed that 5 volatile compounds such as o-cymene, dipentene, γ -terpinene, heneicosane, and 2,4-di-tert-butylphenol had the higher molecular interaction according to the binding energy. But those volatile compounds had no hydrogen bonding, which indicated that antibacterial mechanism might be done other than inhibition of DHPS mechanisms. Interestingly, one of the volatile compounds 2,5-piperazinedione showed less binding energy (-5.20 kcal/mol) with greater hydrogen bonding (PRO15; THR53; VAL17; GLY51) (Fig. 5) comparing to other volatiles tested in the present study.

Conclusion

In the present study, endophytic fungus (*Paraconiothyrium brasiliense* MT269522) was isolated from the fruit of *Capsicum annum* L. and reported for the first time. The EAE of *P. brasiliense* (NCBI accession no. MT269522) contains bioactive volatile compounds such as γ -terpinene, 4-carvomenthenol, heneicosane, 2,5-piperazinedione. Therefore, EAE of *P. brasiliense* significantly inhibits DPPH, ABTS⁺, and ferric ion free radical formation. In addition, the EAE did not show any toxicity to HEK293 cells, but notable cytotoxicity to cancer cells (PC3). Interestingly, the EAE of *P. brasiliense* showed broad-spectrum antibacterial activity against human Gram-positive and Gram-negative bacterial pathogens as equal to standard antibiotic tetracycline hydrochloride. Also, molecular docking analysis confirmed 2,5-piperazinedione had the considerable interaction with DHPS enzyme thereby induced antibacterial activity like sulfonamide class of antibiotic. We conclude that EAE of endophytic fungus (*P. brasiliense*) could be a potential source for the discovery of antioxidant, anticancer, and antimicrobial molecules.

Declarations

Ethics Approval

This article does not contain any studies with animals or human participants.

Consent to Participate

The authors agreed to participate in this work.

Consent for Publication

The authors agreed to publish this work.

Author Contributions

A. Sathiyaseelan: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing - original draft, Writing - review & editing. K. Saravanakumar: Validation, Data curation, Writing - review & editing. A.V.A. Mariadoss: Formal analysis, Data curation, Software. M-H. Wang: Funding acquisition, Project administration, Resources, Supervision, Validation, Writing - review & editing.

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Conflict of Interest

The authors declare no competing interests.

Availability of data and materials

The data generated and analyzed during the current study are available from the corresponding author on reasonable request.

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Tables

Due to technical limitations, tables are only available as a download in the Supplemental Files section.

Figures

Figure 1

The culture morphology of *P. brasiliense* on PDA medium – A, and phylogenetic tree constructed using neighbour-joining method – B. ▲ represent the isolated sequence from *Capsicum annum*.

Figure 2

GC-MS spectrum of EAE of *P. brasiliense*. The inner image showed the existence volatile molecules in the EAE-PB.

Figure 3

Cell viability of ethyl acetate extract of *P. brasiliense* treatment. HEK 293 cells – A; PC3 cells – B.

Figure 4

Apoptotic cell analysis. The IC₅₀ concentration of EF-EA extract treated PC3 cells. Stained with propidium iodide (PI), 4',6-diamidino-2-phenylindole (DAPI), rhodamine 123 (RH-123), and 2',7'-Dichlorofluorescein diacetate (DCFH-DA).

Figure 5

Antibacterial activity of ethyl acetate extract of *P. brasiliense*. A - *Bacillus cereus*; B - *Staphylococcus aureus*; C - *Listeria monocytogenes*; D - *Escherichia coli*, E- *Salmonella enterica*. a - 125 ($\mu\text{g/mL}$), b - 250 ($\mu\text{g/mL}$), c - 500 ($\mu\text{g/mL}$), d - 1000 ($\mu\text{g/mL}$), e - TCH-20 ($\mu\text{g/mL}$).

Figure 6

2D View from a molecular modeling study of the minimum-energy structure of the complex of piperazinedione (A) and 4-carvomenthol (B) redocked in DHPS (PDB ID: 5JQ9).

Supplementary Files

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