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Antioxidant and antibacterial activity of extracts and compounds from endophytic fungi isolated from roots of *Physalis angulata* and their combination effects

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ABSTRACT: Natural products sourced from endophytic fungal are recognized as one of the most important resources for drug discovery and molecular diversity. Utilizing combinations of extracts or pure compounds can produce synergistic effects, offering strong pharmacological efficacy at relatively low concentrations. *Physalis angulata L.* is a medicinal plant traditionally used by people globally. The endophytic fungi associated with this plant represent a valuable, yet underexplored, source of bioactive compounds. This study aimed to identify extracts and pure compounds from endophytic fungi isolated from the roots of *P. angulata* that exhibit antioxidant and antibacterial activities, as well as to explore their combined effects. The endophytic fungi isolates used in this study were obtained from *P. angulata* roots stored in the laboratory as stock cultures. Each isolate was re-identified morphologically for purity and cultivated in Potato Dextrose Broth (PDB) media for 4 weeks at room temperature under static conditions. Liquid culture was extracted in ethyl acetate and evaporated. Antioxidant and antibacterial activities were tested for each endophytic fungal extract, followed by the isolation of active compounds from the selected extracts. The chemical structures were elucidated using spectroscopic techniques, such as 1D and 2D NMR. The endophytic fungi responsible for producing bioactive compounds were identified through molecular analysis. Combination effects were examined on both extracts and pure compounds exhibiting antioxidant and antibacterial activities. The extract of Trichoderma virens endophytic fungi, which produced two bioactive compounds, demonstrated the highest antioxidant and antibacterial activities. Spectroscopic analysis indicated that the two compounds were 10-hydroxy-benzoisochromen-1-one (1) and 7-hydroxy-benzochromen-6-one (2). Molecular identification and phylogenetic analysis of the selected endophytic fungi showed a high similarity to *Trichoderma virens*. The best combination effect with strong antioxidant activity was found in the CA4+CA6 blend (test 3). The antioxidant activity of compounds 1, 2, and their combination products exhibited weak antioxidant activity. The highest antibacterial activity (≥ 95%) for the combination product was derived from synergistic effects (27.8%) and additive effects (13.9%). Compound 1 had strong antibacterial activity compared to compound 2 and their combination products. Thus, for the development of *Trichoderma virens* as a source of medicinal substances, extract combinations are more efficient than pure compound combinations.

KEYWORDS: Antioxidant; antibacterial; combination effects; endophytic fungi; *Physalis angulata*.

1. INTRODUCTION

Free radicals can induce oxidative stress in the body, which heightens the risk of chronic conditions such as diabetes, atherosclerosis, inflammation, hypertension, cardiovascular diseases, neurodegenerative disorders, and cancer [1–4]. Antioxidants can prevent oxidative stress and inhibit its negative impact [5,6]. Natural sources of

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antioxidants, including vegetables, fruits, spices, and medicinal plants, are well-known for their rich content of vitamin C, vitamin E, and phenolic compounds.

Bacterial infections, such as bacillary dysentery, tuberculosis, pneumonia, sepsis, typhoid, diarrhea, and tetanus, are common across all age groups. Antibiotics, including penicillin, cephalosporins, macrolides, fluoroquinolones, and others, are drugs that combat microbes and are regarded as a groundbreaking advancement in medical history [7–9]. However, the overuse of antibiotics promotes changes in bacteria that enable them to develop resistance to specific classes of these drugs, resulting in antimicrobial resistance (AMR). This means that infections caused by resistant bacterial strains become difficult to treat with existing antibiotics. The rise of AMR complicates infection treatment, often leading to prolonged infections and treatment failures [10–12].

The effects of free radicals and infectious diseases caused by pathogenic bacteria, along with bacterial resistance to available antibiotics, have posed challenges in the field of medicine [13,14]. Therefore, urgent action is needed to address these problems by searching for and developing new sources of antioxidant and antibacterial compounds to complement the current available drugs. To discover these bioactive compounds, several strategies must be employed, including the exploration of endophytic fungi. These microscopic organisms reside within plant tissues—such as leaves, fruits, seeds, stems, and roots—for a certain period, forming colonies without causing harm to the host. Often, they engage in a mutually beneficial relationship with the plant. Endophytic fungi are promising and abundant sources of natural products with diverse chemical structures, high biodiversity, and various interesting bioactivities.

One of the medicinal plants selected as a host for endophytic fungi is *Physalis angulata* L. This plant, which has an ethnobotanical history related to its medicinal uses, presents a great opportunity to discover endophytic fungal as a source of new compounds which have bioactivity. Studies have demonstrated that *P. angulata* possesses significant cytotoxic effects against cancer cell lines, including breast and lung cancer, through mechanisms involving apoptosis induction. Additionally, it exhibits immunomodulatory activity, making it a promising candidate in the development of treatments for autoimmune diseases and chronic inflammatory conditions. *Physalis angulata* has been used traditionally to treat diabetes, asthma, kidney and bladder problems, inflammation, hepatitis, gout, skin infections, cancer, digestive issues, and more [15–17]. The plant has been experimentally tested for its antibacterial, anti-inflammatory, anticancer, antiparasitic, antinociceptive, antimalarial, antileishmanial, immunosuppressive, diuretic, antiasthmatic, antipyretic, analgesic, anti-inflammatory, anticoagulant, antileukemia, and detoxification properties [18–21]. The active compounds contained in *P. angulata* include steroids, alkaloids,flavonoids, terpenoids, saponins, glycosides, carotenoids, tannins,physalins, and withanolides, especially the C28 steroidal lactone series [22–25]. The medicinal uses of this plant are closely related to its endophytic fungal population, as these fungi may produce bioactive compounds beneficial for health. The presence of endophytes could lead to the discovery of novel bioactive compounds with potential therapeutic applications. By understanding the interactions between the plant and its endophytes, researchers can not only enhance the yield of valuable compounds but also uncover new pathways for bioprospecting [26].

Plant extracts contain many antioxidants and antimicrobial constituents that provide various antioxidant and antimicrobial activities. Enhanced or broad-spectrum activity can result from the interactions of two or more extracts/compounds in combination [27,28]. When combining extracts or compounds, four possible effects can occur: indifference [the combination product has the same effect as the most active individual component], additive effect [the combination product's effect equals the amount of the individual components], synergism [the combination product produces a greater effect than the sum of the individual components], and antagonism [the combination product has a weaker effect than the most effective individual component] [29]. This study applies the same principle to investigate the combination effects of extracts and pure chemical compounds from endophytic fungal isolated from the roots of *Physalis angulata* on their individual antioxidant and antibacterial activities.

2. RESULTS

2.1 Rejuvenation of Endophytic Fungi and Morphological Identification

The rejuvenated endophytic fungi were obtained by re-growing the purified isolates in PDA media and incubating them for 5-7 days at room temperature. A total of 10 endophytic fungi isolates from *P. angulata* roots were successfully rejuvenated (codes CA1 - CA10). All isolates exhibited diverse colony characteristics (Figure 1). The macroscopic and microscopic characteristics of each isolate are shown in Table 1 and Table 2.

Table 1. Characteristics' colony of endophytic fungi from the root of *Physalis angulata* L.

Code	Surface colony	Reverse colony	Texture	Topography	Pattern	Exudate drops	Radial line	Concentric cyrcle
CA1	Green to yellow	White	granular	Raised	Zonate	-	-	√
CA2	Grey white	Reddish brown to red	Cottony	Raised	Zonate	-	-	-
CA3	Grey	Dark grey	granular	Raised	Zonate	-	-	√
CA4	White	Pale cream	Cottony	Raised	spread	-	-	-
CA5	Grey	Black to grey	Cottony	Raised	Spread	-	-	-
CA6	White	Black	Cottony	Flat	spread	-	-	-
CA7	Black White	Cream	Powdery	Umbonate	Zonate	-	-	-
CA8	Grey white	Dark cream to cream	Powdery	Raised	Spread	-	-	-
CA9	White	Black-white	Powdery	Raised	Spread	-	-	-
CA10	White	Black	Powdery	Raised	Spread	-	-	-

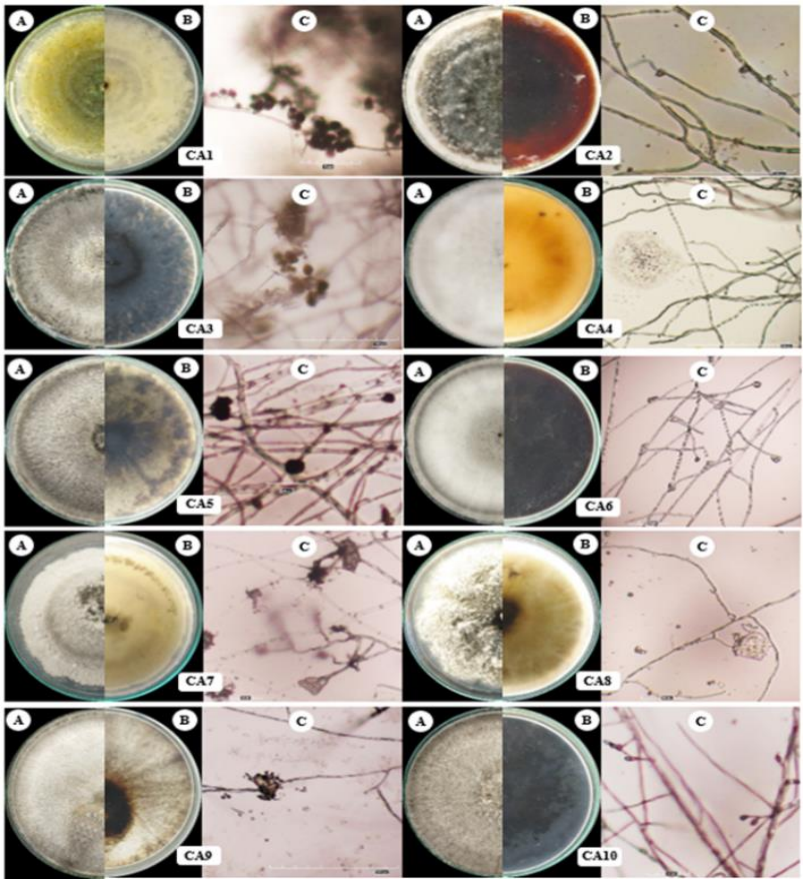


Figure 1. Characteristics morphology of endophytic fungus colonies isolated from **Physalis angulata** roots. A. Macroscopic characteristics (front view); B. Reverse view; C. Microscopic characteristics.

Table 2. Microscopic characteristics of endophytic fungi from the root bark of *Physalis angulata*

Isolates	Spora	Shape	Hyphae	Specific characteristic	Genus
CA1	conidia	Globose	septate	Conidiophores are hyaline, erect, and branched	*Trichoderma* sp.
CA2	Sporangio-spores	ovoid	septate	Papulaspores formed on and in agar media or aerially	*Papulaspora* sp.
CA3	Conidia	Globose	septate	Conidiophores hyaline, erect, and branched	*Trichoderma* sp
CA4	Sporangio-spores	globose	septate	Sporangiophores pale brown, erect, simple or branched	*Mortierella* sp.
CA5	Sporangio-spores	Globose	septate	Papulaspores formed on and in agar media or aerially	*Papulaspora* sp
CA6	Conidia	Globose	septate	Conidiophores brown, branched, bearing spore masses apically	*Phialophora* sp
CA7	Conidia	Cylindrical	septate	Conidiophores hyaline, mostly branched with verticillate phialides bearing terminal spore masses	*Verticillium* sp
CA8	Papulo-spores	Globose	septate	Papulaspores formed on and in agar media or aerially	*Papulaspora* sp
CA9	Papulo-spores	Globose	septate	Papulaspores formed on and in agar media or aerially	*Papulaspora* sp
CA10	Conidia	Ellipsoidal	septate	Conidiophores are not well differentiated from conidia, appearing indistinct or short, and cylindrical if present	*Trichocladium* sp.

Table 1 and Table 2 described the morphological characteristics of each endophytic fungal isolate from the roots of *P. angulata*. Six genera of endophytic fungi were found, namely Trichoderma, Papulaspora, Mortierella, Phialophora, Verticillium, and Trichocladium. The endophytic fungi were identified based on their characteristics that appeared.

2.2 Bioactivity of Endophytic Fungi Extracts Isolated from *Physalis angulata*

Endophytic fungal isolated from *P. angulata* showed interesting bioactivity, as evidenced by the varying categories of antibacterial and antioxidant activities, ranging from weak to very strong (Table 3). The CA3 extract of endophytic fungi showed the best antibacterial activity among other fungal isolate extracts, with strong activity percentages for all four test bacteria, while its antioxidant activity was still in the moderate category. However, compared to other extracts, the antioxidant activity of the CA3 extract was among the best, categorized as moderate.

2.3 Molecular Identification of Endophytic Fungi Isolated from *P. angulata*

Based on the bioactivity test results, the CA3 isolate demonstrated the most promising outcomes. Consequently, molecular identification of the CA3 endophytic fungal isolate was pursued using molecular techniques. The results of this identification are presented in Figure 2, which depicts a phylogenetic tree showing that the CA3 isolate is classified within the *Trichoderma virens* species.. The sequence of the CA3 isolate is as follows:
TAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTACCGAGTTTACAACTCCCAAACCCAATGT
GAACGTTACCAAACCGTTGCCTCGGCGGGATCTCTGCCCCGGGTGCGTCGCAGCCCCGGACCAAGGCG
CCCGCCGGAGGACCAACCAAAACTCTTATTGTATACCCCCTCGCGGGTTTTTTACTATCTGAGCCATCTC
GGCGCCCCTCGTGGGCGTTTCGAAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCG
ATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGA
ACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACCCTCGAACCCC
TCCGGGGGGTCGGCGTTGGGGATCGGCCCTTTACGGGGCCGGCCCCGAAATACAGTGGCGGTCTCGCC
GCAGCCTCTCCTGCGCAGTAGTTTGCACACTCGCATCGGGAGCGCGGCGCGTCCACAGCCGTTAAACA
CCC.

Table 3. Antibacterial activity percentage and IC_{50} of endophytic fungi extract isolated from the root of *Physalis angulata* L with ascorbic acid and tetracycline as positive control

Samples	Extract	Genus	Antibacterial activity (%)				Antioxidant Activity IC_{50} (µg/mL)
			S. typhi	*E. coli*	*B. subtilis*	*S. aureus*	
Endophytic fungi	CA1	*Trichoderma* sp.	50.00±0.92 *	55.08±1.51 **	47.83±0.62 *	53.85±0.03 **	198.28 **
	CA2	*Papulaspora* sp.	70.83±0.71 ***	85.72±1.42 ***	69.57±0.89 **	80.77±0.71 ***	> 1000 na
	CA3	*Trichoderma* sp.	79.17±0.89 ***	90.44±1.71 ***	73.91±0.29 ***	96.15±2.05 ***	118.39 **
	CA4	*Mortierella* sp.	58.33±0.71 **	60.09±0.73 **	69.57±0.71 **	76.92±0.47 ***	377.38 **
	CA5	*Papulaspora* sp.	37.50±0.21 *	45.13±0.71 *	39.13±0.96 *	34.62±0.71 *	> 1000 na
	CA6	*Phialophora* sp.	50.00±0.20 *	60.21±0.30 **	65.22±1.41 **	50.40±0.68 *	123.98 **
	CA7	*Verticillium* sp.	79.17±1.34 ***	85.11±1.40 ***	69.57±1.65 **	69.23±0.25 **	135.73 **
	CA8	*Papulaspora* sp.	54.17±0.41 **	75.20±1.03 ***	60.87±1.21 **	57.69±0.32 **	107.3 **
	CA9	*Papulaspora* sp.	62.50±1.45 **	65.01±0.14 **	56.52±0.07 **	61.84±0.88 **	65.3 ***
	CA10	*Trichocladium* sp.	66.67±1.02 **	98.30±0.71 ***	97.08±0.71 ***	60.54±1.41 **	976.49 *
Positive control	Tetracycline		100±2.20 ***	100±1.80 ***	100±2.91 ***	100±2.24 ***	-
	Ascorbic Acid		-	-	-	-	10.33 ****

Note: Antibacterial activity: ***) strong>70%; **) moderate 50%-70%; *) weak<50%; Antioxidant activity IC_{50}(µg/mL): ****very strong< 20µg/mL ***strong< 100 µg/mL; **moderat 100-500µg/mL; * weak>500µg/mL;[na] inactive

2.4 Isolation and Identification of Compound 1 and Compound 2

Ethyl acetate extract CA3-A (2g) was pre-absorbed with 2 g of silicagel 60 (70-230 mesh) and separated using column chromatography (CC) with enhancing polarity eluents, that were n-hexane:EtOAc (10:0→0:10) and EtOAc:methanol (10:0→0:10). The eluates were collected in 67 vials of 10 mL each, evaporated, and analyzed by TLC to observe the spot patterns. Eluates with similar spot patterns were combined into one fraction, resulting in five fractions (F1-F5). Fraction F3, which showed a major spot pattern with pale yellow solid, was re-chromatographed with n-hexane:EtOAc (7:3→2:8) to produce four subfractions (F3.1-F3.4). SubfractionF3.3 was rinsed with a solvent mixture of n-hexane:EtOAc (1:1) to obtain compound 1 (CA3-A2) in the form of a pale yellow solid weighing 41.3mg. Fraction F4 also showed a major spot pattern with yellow solid and was further separated by re-chromatography with n-hexane:EtOAc (6:4→0:10), resulting in four subfractions (F4.1-F4.4). Subfraction F4.2 was rinsed with a solvent mixture of n-hexane:EtOAc (1:1) to obtain compound 2 (CA3-B1) in the form of yellow solid weighing 33.1 mg.

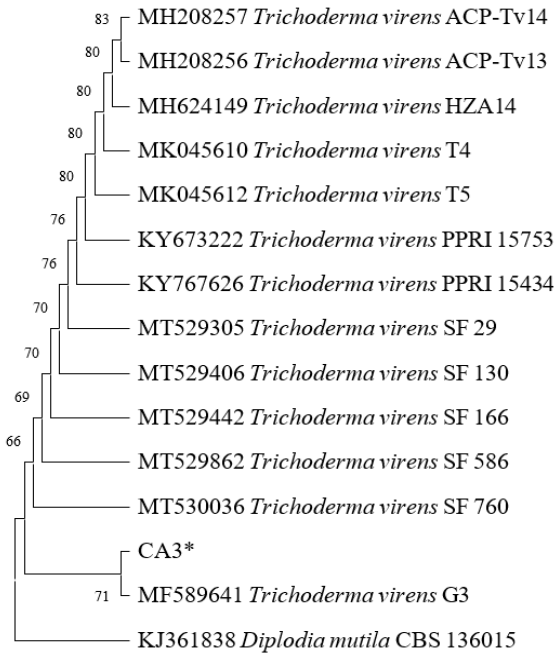


Figure 2. Phylogenetic tree of the CA3 isolate

2.4.1 Compound 1

The ^{1}H-NMR spectrum (700 MHz CDCl3) (Figure 3A) shows eight proton signals, consisting of seven aromatic and vinylic proton signals (δ_H 7.99 – 8.99 ppm) and one hydroxyl proton signal (δ_H 15.59 ppm). The multiplicity of two vinylic proton signals is a doublet with a coupling constant of J = 7.0 Hz, two aromatic proton doublets with J = 8.4 ppm, and two aromatic proton signals as multiplets. This indicates that compound 1 has a three-ring structure, including aromatic and heteroatom rings with seven ortho-positioned sp^2 protons. The ^{13}C-NMR spectrum (176 MHz CDCl3) (Figure 3B) shows 13 carbon signals, all in the δ_C > 100 ppm region as sp^2 carbons. The carbon signal at δ_C 166.2 ppm is characteristic of a ketone carbonyl carbon. Based on the information obtained from the ^{1}H-NMR and^{13}C-NMR spectra, compound 1 is composed of three rings (A, B, and C), namely two aromatic rings and one lactone ring. The hydroxylgroup is attached to the aromatic ring, forming a hydrogen bond with the lactone carbonyl group.

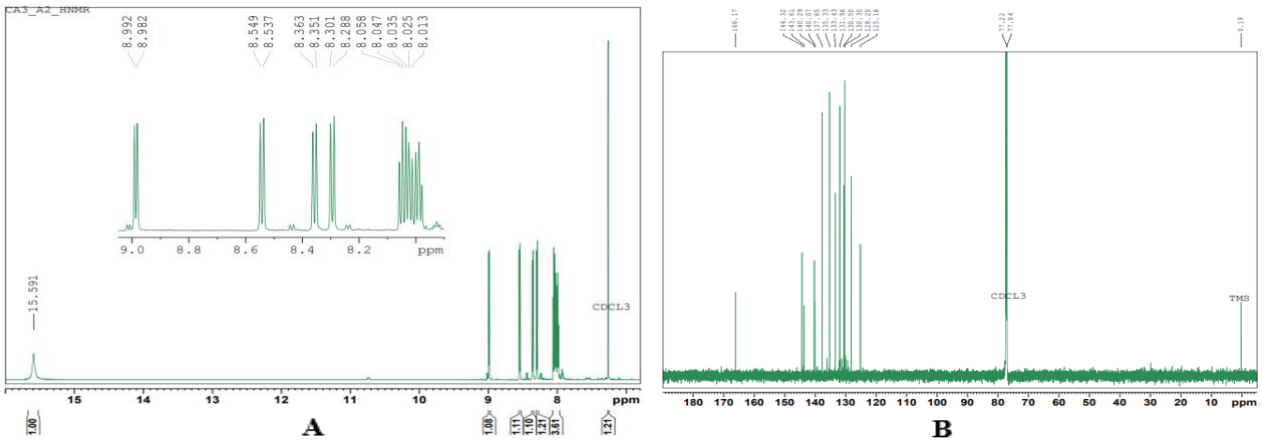


Figure 3. The ^{1}H-NMR (A) and ^{13}C-NMR (B) spectra of compound 1

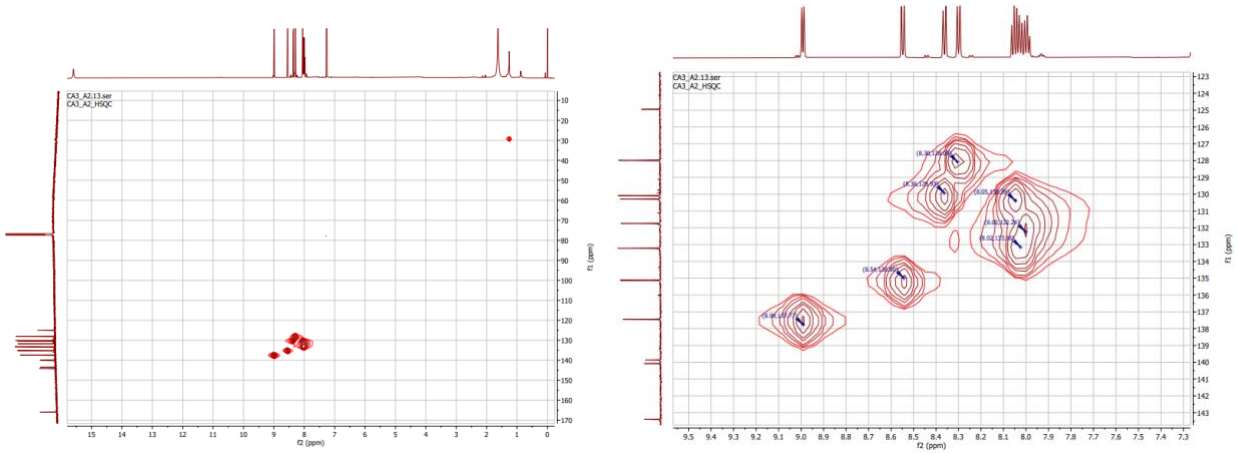


Figure 4. The HSQC Spectral of Compound 1

Direct correlation between carbon and proton atoms can be seen in the HSQC spectrum (Heteronuclear Single Quantum Correlation). The HSQC spectrum of compound 1 (Figure 4) shows seven sp2 proton-carbon correlations. There is one proton signal that is not bonded to a carbon atom, indicating that the proton belongs to a hydroxyl group. Thus, it is known that compound 1 has seven sp2 tertiary carbons and six sp2 quaternary carbons, forming a three-ring framework, including two aromatic rings and one lactone ring.

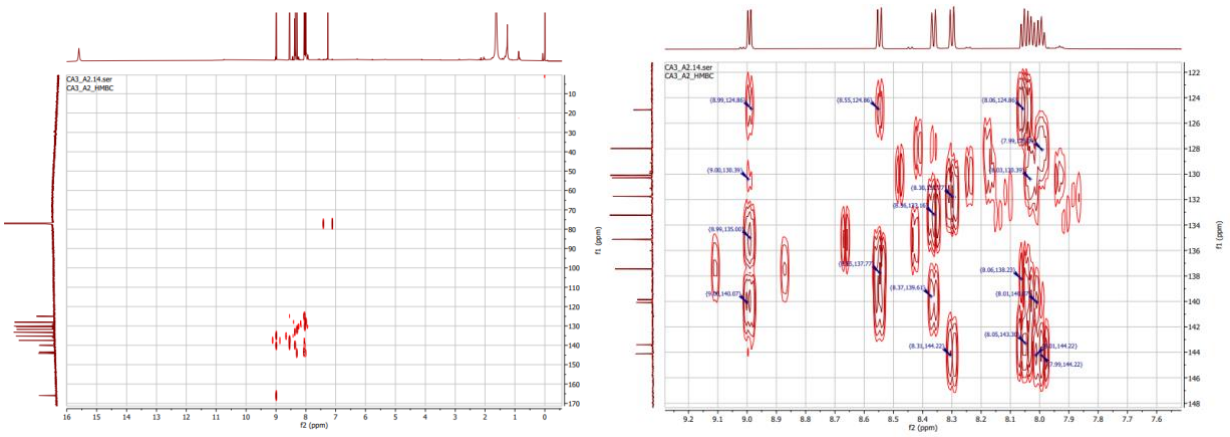


Figure 5. The HMBC Spectral of Compound 1

The correlation between protons and neighboring carbons with a distance of two to three bonds was determined through the HMBC spectrum. The HMBC spectrum of compound 1 (Figure 5) explained that the two aromatic protonsignals at δ_H 8.99 (^{1}H; d; 7.0 Hz) and 8.05 ppm (^{1}H; d; 7.0 Hz) have the same coupling constant (J = 7 Hz), correlating with each other and with the same carbon signal at δ_C 125.1 ppm. Additionally, the proton at δ_H 8.99 ppm also correlates with the lactone carbonyl carbon at δ_C 166.2 ppm over three bonds. This indicated that there is a lactone ring in compound 1 with two adjacent sp2 methine protons. Furthermore, there are other correlations that reinforce the presence of two fused benzene rings. The aromatic proton at δ_H 8.54 (^{1}H; d; 8.4 Hz) correlates with the quaternary carbons at δ_C 125.1 and 143.6 ppm, where these carbon signals are the connecting carbons between the cyclic ester ring (δ_C 125.1 ppm) and the other benzene ring (δ_C 143.6 ppm). The aromatic proton at δ_H 8.30 (^{1}H; d; 8.4 Hz) ppm correlates with the carbons at δ_C 133.4 and 140.0 ppm, which are the connecting quaternary carbons of the benzene ring. This indicates that the two protons are ortho-coupled on the same benzene ring with a tetrasubstituted ring structure. The proton at δ_H 8.36 (^{1}H; d; 9.1 Hz) is split into a doublet by the ortho-positioned proton. Next, theprotons at δ_H 8.02 (^{1}H; m) and 7.99 (^{1}H; m) are split into multiplets by the ortho-para and ortho-ortho protons, resulting in a multiplet multiplicity.

Analysis of the H-NMR, C-NMR, HSQC, and HMBC spectra revealed that compound 1 consists of two fused benzene rings (A and B) and a fused lactone ring (C). A hydroxylgroup is attached to ring A, forming a chelate with the lactone carbonyl group. Consequently, compound 1 is identified as 10-hydroxy-benzoisochromen-1-one. The 1D and 2D NMR spectral data for compound 1 are detailed in Table 4, while its molecular structure, including carbon atom numbering, proton and carbon-chemical shifts, and HMBC correlations, is illustrated in Figure 6.

Table 4. 1D and 2D NMR spectral data of compound 1

No. C	δ_C ppm 1	δ_H ppm (ΣH. Multiplicity, Hz) 1	HMBC 1
C1	166.2	-	-
C3	137.6	8.99 (^{1}H; d; 7.0 Hz)	125.1; 135.3; 140.3; 166.2
C4	130.5	8.05 (^{1}H; d; 7.0 Hz)	125.1; 137.6; 143.6
C5	135.3	8.54 (^{1}H; d; 8.4 Hz)	125.1; 137.6
C6	128.2	8.30 (^{1}H; d; 8.4 Hz)	133.4; 140.0
C7	133.4	8.02 (^{1}H; m)	130.3; 140.0
C8	131.9	7.99 (^{1}H; m)	128.2; 144.3
C9	130.3	8.36 (^{1}H; d; 9.1 Hz)	131.9; 143.6
C10	144.3	-	-
C11	140.0	-	-
C12	143.6	-	-
C13	125.1	-	-
C14	140.3	-	-
C10-OH	-	15.59 (1H; s)	-

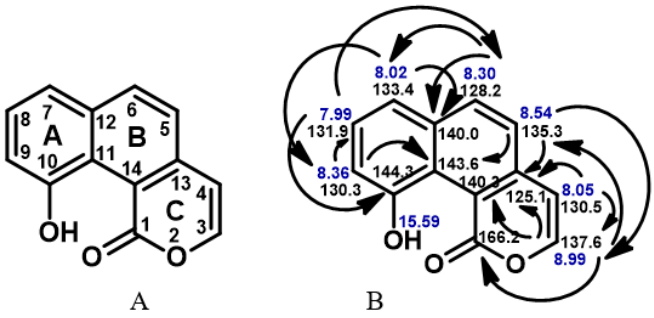


Figure 6. Structure of compound 1, 10-hydroxy-benzoisochromen-1-one with carbon atom numbering (A), proton chemical shift placement, carbon, and HMBC correlation (B).

2.4.2 Compound 2 (CA3-B1)

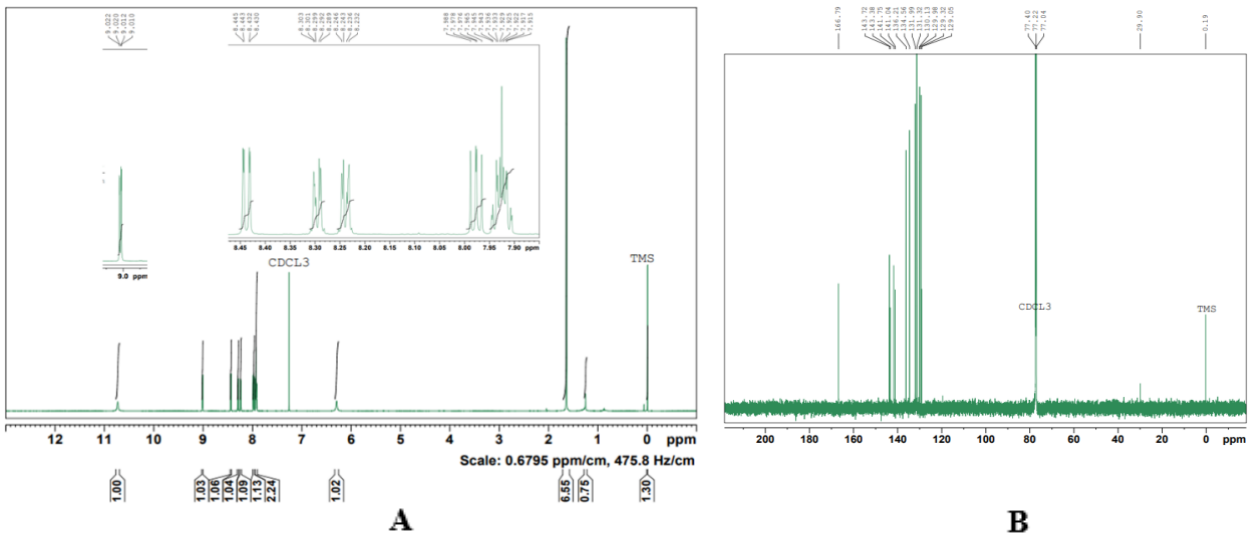


Figure 7. The ^{1}H-NMR (A) and ^{13}C-NMR (B) spectra of compound 2

The ^{1}H-NMR spectrum (700 MHz CDCl3) (Figure 7A) shows eight proton signals, consisting of seven aromatic protonsignals(δ_H 7.92 – 9.03 ppm) and one hydroxylproton signal at δ_H 10.73 ppm (^{1}H; s). The multiplicity of two

proton signals at δ_H 9.03 (^{1}H dd J= 7; 14 Hz) and 7.99 ppm (^{1}H dd J= 8.75; 7 Hz) is a doublet of doublets with the same couplingconstant of 7.0 Hz, indicating that the two protons are ortho-positioned. The proton at δ_H 8.45 (^{1}H dd J= 8.75; 14 Hz) has a doublet of doublets multiplicity with the same coupling constant as the proton at δ_H 7.99 ppm (8.75 Hz ortho-position) and the proton at δ_H 9.03 ppm (14 Hz meta-position). Thus, it is known that these three protons are sequentially positioned on a trisubstituted benzene ring. Next, the proton at δ_H 8.23 (^{1}H dd J= 7.35; 2.1 Hz) with a doublet of doublets multiplicity is ortho-coupled with theproton at δ_H 7.93 (^{1}H m) and meta-coupled with the proton at δ_H 7.92 (^{1}H m). The last aromatic proton at δ_H 8.31(^{1}H dd J= 8.05; 1.4 Hz) is ortho-coupled with theproton at δ_H 7.92 (^{1}H m) and metacoupled with the proton at δ_H 7.93 (^{1}H m). Thus, it is known that the four aromatic protons are sequentially positioned on a disubstituted aromatic ring.
The ^{13}C-NMR spectrum (176 MHz CDCl3) (Figure 7B) shows 13 carbon signals, all in the δ_C > 100 ppm region as sp2 carbons. The carbon signal at δ_C 166.8 ppm is characteristic of a ketone carbonyl carbon. Based on the information obtained from the ^{1}H-NMR and^{13}C-NMR spectra, compound 2 is composed of three rings (A, B, and C), namely two aromatic rings and one lactone ring. The hydroxylgroup is attached to the aromatic ring, forming a hydrogen bond with the lactone carbonyl group.

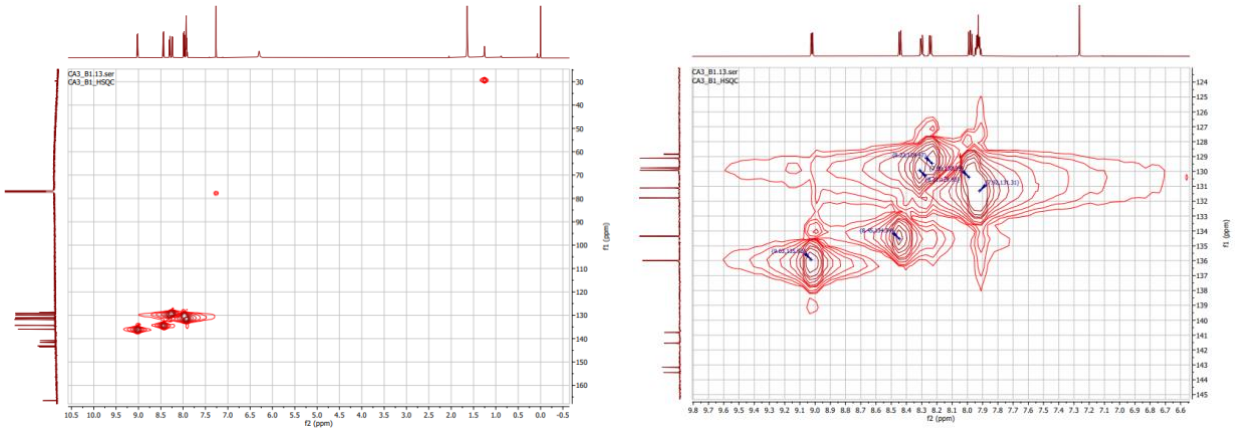


Figure 8. The HSQC Spectral of Compound 2

Direct correlation between aromatic carbon and proton atoms can be seen in the HSQC spectrum (Heteronuclear Single Quantum Correlation). The HSQC spectrum of compound 2 (Figure 8) shows seven aromatic proton-carbon correlations. There is one proton signal that is not bonded to a carbon atom, indicating that the proton belongs to a hydroxyl group. Thus, it is known that compound 2 has seven aromatic carbons, five quaternary aromatic carbons, and one lactone carbonyl carbon, forming a three-ring framework, including two aromatic rings and one lactone ring.

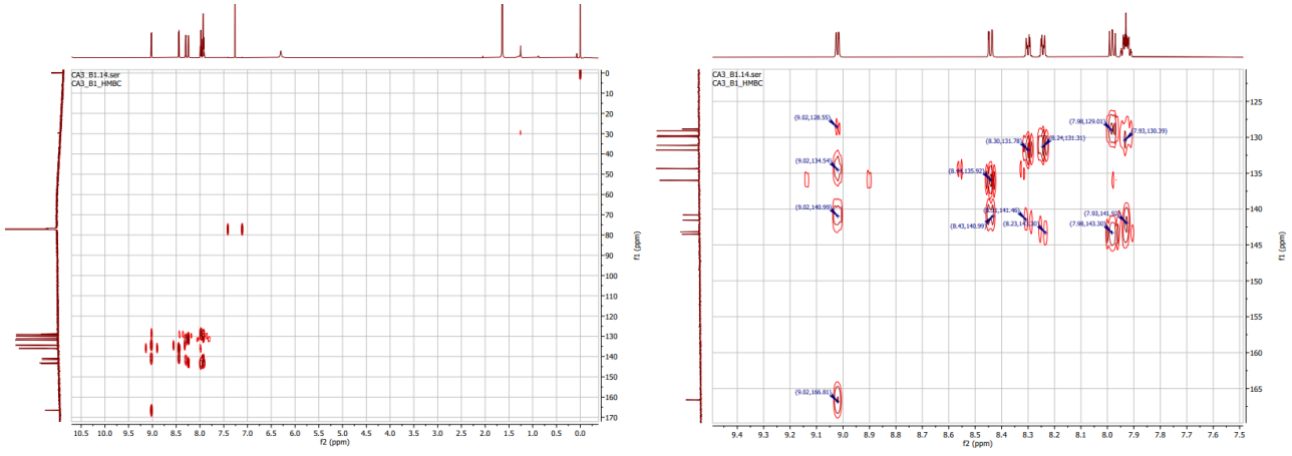


Figure 9. The HMBC Spectral of Compound 2

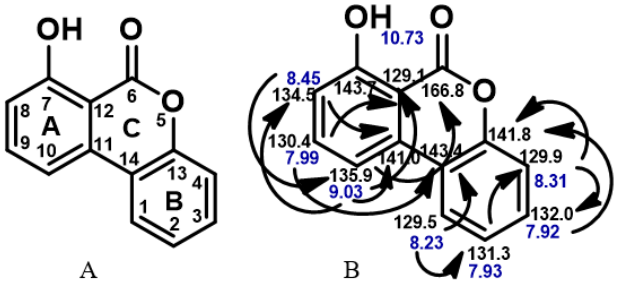
The correlation between protons and neighboring carbons with a distance of two to three bonds was determined through the HMBC spectrum (Heteronuclear Multiple-Bond Correlation). The HMBC spectrum of compound 2(Figure 9) shows that the two aromatic proton signals at δ_H 9.03 (^{1}H dd J= 7.0; 14 Hz) and 7.99 (^{1}H dd J= 8.75; 7.0 Hz) have the same coupling constant (J = 7 Hz) and correlate with the same carbon signal at δ_C 129.1 ppm.

Theproton at δH 9.03 ppm also correlates over two and three bonds with the carbons at δ_C 134.5, 141.0 ppm, and has long-range coupling with the lactone carbonyl carbon at δ_C 166.8 ppm. Additionally, the proton at δ_H 8.45 ppm (^{1}H dd J= 8.75; 14 Hz) correlates over three bonds with the carbon at δ_C 135.9 ppm and has long-range coupling with the quaternary aromatic carbon at δ_C 141.0 ppm. This indicates that there are three sequential aromatic protons on ring A, experiencing doublet of doublets multiplicity from ortho-meta and ortho-ortho coupling. Next, it is known that aromatic ring A is fused with lactone ring C through the correlation of these protons to the connecting carbons at δ_C 129.1 and 141.0 ppm. Furthermore, the proton at δ_H 8.23 (^{1}H dd J= 7.35; 2.1 Hz) correlates over two bonds with the carbons at δ_C 131.3 and 143.4 ppm, and the doublet of doublets multiplicity is obtained from ortho-coupling with the proton at δ_H 7.93 (^{1}H m) and meta-coupling with the proton at δ_H 7.92 (^{1}H m). The aromatic protons at δ_H 7.93 (^{1}H m) and 7.92 (^{1}H m) each correlate over two bonds with the carbons at δ_C 129.9 and 141.8 ppm. Lastly, the proton at δ_H 8.31 ppm (^{1}H dd J= 8.05; 1.4 Hz) correlates with the carbons at δ_C 132.0 and 141.8 ppm, which are oxy-lactone carbons. Thus, it is known that these four aromatic protons are sequentially positioned on ring B, which is fused with lactone ring C.

Based on the analysis of the H-NMR, C-NMR, HSQC, and HMBC spectra, it is known that compound 2 has two aromatic rings (A and B) and one lactone ring (C). Aromatic rings A and B are each fused with lactone ring C. A hydroxyl group is attached to ring A, which chelates with the lactone carbonyl group. Thus, compound 2 is identified as 7-hydroxy-benzochromen-6-one. The 1D and 2D NMR spectral data of compound 2 are presented in Table 5. The molecularstructure of compound 2, along with carbon atom numbering, the proton and the carbon-chemical shifts, andHMBC correlations, are presented in Figure 10.

Table 5. 1D and 2D NMR spectral data of compound 2

No. C	δ_C ppm 2	δ_H ppm (ΣH. Multiplicity, Hz) 2	HMBC 2
1	129,5	8,23 (1H, dd, J= 7.35; 2.1 Hz)	131,3; 143,4
2	131,3	7,93 (1H, m)	129.9
3	132,0	7,92 (1H, m)	141,8
4	129,9	8,31 (1H, dd, J= 8.05; 1.4 Hz)	132,0; 141,8
6	166,8		
7	143,7		
8	134,5	8,45 (1H, dd, J= 8.75; 1,4 Hz)	135,9; 141,0
9	130,4	7,99 (1H, dd, J= 8,75; 7.0 Hz)	129,1; 143,4
10	135,9	9,03 (1H, dd, J= 7.0; 1,4 Hz)	129,1; 134,5; 141,0; 166,8
11	141,0		
12	129,1		
13	141,8		
14	143,4		
7-OH		10.73 (1H; s)	



A B

Figure 10. Molecular structure of compound 2: 7-hydroxy-benzochromen-6-one with carbon atom numbering (A), proton and carbon chemical shifts, and HMBC correlations (B)

2.5 Combination Effects

2.5.1 Combination effect on antioxidant activity

Each endophytic fungal extract was combined with two or three other extracts in a 1:1 ratio, and The antioxidant activity was evaluated by calculating the percentage of inhibition. The results, showing % inhibition for both individual extracts and their combinations, are presented in Figure11.

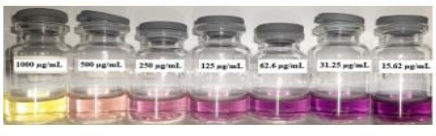
The 1,1-diphenyl-2-picrylhydrazyl (DPPH) method is based on the principle that an antioxidant compound donates a hydrogen atom to the DPPH radical, reducing it to a non-radical form. This reduction causes DPPH to lose its characteristic purple color. The antioxidantactivity of compounds is assessed by their ability to inhibit DPPH absorption, measured as a percentage of inhibition at a wavelength of 517 nm. DPPH strongly absorbs at this wavelength with a dark violet hue, representing a collection of free radicals. When an antioxidant compound donates a hydrogen atom to DPPH, the intensity of the purple color diminishes, fading to yellow due to the picryl group's presence. The color intensity change correlates with the antioxidant's strength. The DPPH radical cavenging activity is determined by measuring the absorbances of the remaining DPPH radicals using a UV-visspectrophotometer at λmax 517 nm, with a more pronounced yellow color indicating stronger antioxidant activity.

The combination effect of endophytic fungal extracts in tests 1-9 on antioxidant activity (Figure 11) showed that seven tests (77.8%) were additive effects, one test (11.1%) was an indifference effect, and one test (11.1%) was a synergism effect. All combination effects in the additive category had a % inhibition value of 71.41 - 89.39%, while the indifference effect had a % inhibition value of 93.86% which was classified as a strong antioxidant at a concentration of 500 µg/mL. Table 3 shows that individual extracts of CA4 and CA6 had a % inhibition value of > 90%.

Note: * indifference ** additive *** synergism **** antagonism

Figure 11. Antioxidant activity (% inhibition at 500 µg/mL) of individual extracts and their combination (1:1/ 1:1:1) of the endophytic fungus *Trichoderma virens*

Table 6. Antioxidant activity (IC_{50} µg/mL) of individual compounds and combinations (1:1) of the endophytic fungal *Trichoderma virens*

Sample	IC_{50} (µg/mL)	Color changes in the concentration series of antioxidant activity tests
Compound 1	262.68	
Compound 2	259.11	
Combination	134.41***	

The antioxidant activity of individual compounds and combination (1:1) from endophytic fungus *Trichoderma virens* (Figure 11) shows that the combination effect is included in the synergistic category. However, both individual compounds and their combination products have IC_{50} values > 100 µg/mL which are classified as weak antioxidants.

2.5.2 Combination effect on antibacterial activity

The antibacterial activity was tested by using the disc paper diffusion method. We also evaluated the antibacterial effects of combining two or three extracts in a 1:1 ratio by measuring the inhibition zones. The results for the antibacterial activity of both individual extracts and their combinations are displayed in Table 7.
The combination effect of endophytic fungal extracts in tests 1-9 on antibacterial activity with four test bacteria (total 36 tests) showed that the most combination effect was additive (47.2%), followed by synergism (33.3%), indifference (11.1%), and antagonism (8.3%). The combination effect of all tests found a strong antibacterial activity category (% antibacterial activity ≥ 70%) of 63.9%, which came from the additive effect and synergism effect of 27.8% each, the indifference effect 5.6%, and 2.8% from the antagonism effect. The highest percentage of antibacterial activity (≥ 95%) for the combination product came from the synergism effect (27.8%) and from the additive effect (13.9%). The antibacterial activity of individual compounds and combinations (1:1) of endophytic fungus *Trichoderma virens* showed that compound 1 has strong antibacterial activity, namely % antibacterial activity ≥ 70% against all test bacteria. Compound 2 has moderate antibacterial activity with % antibacterial activity of 50-70%. The combination product is included in the synergism and additive categories with strong antibacterial activity of 74.9-85.5% against all test bacteria.

3. DISCUSSION

Antioxidants have various biological and pharmacological activities and are considered highly beneficial for nutrition and health. Spices and herbs that act as natural antioxidants are often used in combination. Studies have shown that polyphenolic of green tea exhibit strong synergistic antioxidant activity with tocopherol [30–32]. The total antioxidant properties in vegetables,fruits, and their processed products is the cumulative outcome of synergistic/combination interactions. In binary mixtures, phenolic antioxidants interact to produce synergistic effects, as seen in combinations like rosmarinic acid with quercetin or rosmarinic acid with caffeic-acid [33–36]. These findings provide fundamental information that synergistic effects of extracts or compounds can become a new strength in the pharmaceutical world to reduce unwanted side effects.

Table 7. Antibacterial activity of individual extracts and combination effects of endophytic fungal extracts from *P. angulata* roots at a concentration of 400 µg/dish

Sample	Inhibition Zone (mm) (% Antibacterial Activity)			
	S. aureus	*E. coli*	*S. typhi*	*B. subtilis*
Test 1				
Control (-)	0.00	0.00	0.00	0.00
Control (+)	25.25±1.08 (100)	28.5±1.51 (100)	24.25± 1.83 (100)	25.25± 0.96 (100)
CA1	16.25±0.77 (64.36)	14.75±0.67 (51.75)	13.75± 0.71 (56.70)	20.75± 0.87 (82.18)
CA5	12.5 ± 0.71 (49.50)	11.5 ±0.16 (40.35)	11.25± 0.97 (46.39)	10.5±1.41 (41.58)
CC	16.5±0.81 (65.35)*	12.5±0.80 (43.86)**	13.5± 0.62 (55.67)*	14.5±1.41 (57.43)**
Test 2				
Control (-)	0.00	0.00	0.00	0.00
Control (+)	25.75± 0.98 (100)	25.50±1.06 (100)	26.50± 1.05 (100)	25.75±0.61 (100)
CA2	24.25± 0.81 (94.17)	25.50±0.71 (100)	24.75± 1.31(93.40)	23.50±0.77 (91.26)
CA7	25.75± 0.71 (100)	23.25±0.80 (91.18)	22.25±2.12(83.96)	24.25±1.41 (94.17)
CC	25.25± 0.00 (98.06)**	24.25±1.02 (95.10)**	25.25±0.71(95.28)***	25.25±1.21 (98.06)***
Test 3				
Control (-)	0.00	0.00	0.00	0.00
Control (+)	28.75±2.12 (100)	26.50±0.71(100)	26.75±1.42 (100)	26.25±1.71 (100)
CA3	25.50± 1.10 (88.70)	21.25±2.82 (80.19)	24.50±0.81 (91.59)	23.25± 0.91 (88.57)
CA10	25.75±2.17 (89.57)	23.25±0.90 (87.74)	24.75± 1.20 (92.52)	22.50±2.82 (85.71)
CC	28.25±3.53 (98.26)***	25.50±1.41 (96.23)***	22.75±0.71 (85.05)****	25.50±2.82 (97.11)***
Test 4				
Control (-)	0.00	0.00	0.00	0.00
Control (+)	25.25±0.90 (100)	25.25± 1.31 (100)	25.50± 2.01 (100)	22.75±2.12 (100)
CA4	15.25± 0.46 (60.40)	15.25± 0.43 (60.40)	16.75± 0.32 (65.69)	17.25±1.41 (75.82)
CA8	15.50± 0.52 (61.39)	16.25±1.12 (64.36)	17.75± 0.43 (69.61)	16.25± 0.71 (71.43)
CC	15.25± 0.61 (60.40)**	15.50± 0.71 (61.39)**	15.50±1.41 (60.78)****	17.25± 0.75 (75.82)*
Test 5				
Control (-)	0.00	0.00	0.00	0.00
Control (+)	26.25±2.01 (100)	24.50± 0.96 (100)	27.50±1.41 (100)	25.25±0.71 (100)
CA6	16.25±0.71 (61.90)	14.25± 0.71 (58.16)	15.50±0.71(56.36)	17.25± 0.31 (68.32)
CA9	19.25±1.41 (73.33)	14.75± 0.71 (60.20)	16.25±1.41 (59.09)	16.50±1.24 (65.35)
CC	19.50±1.41 (74.29)*	15.75±0.00 (64.29)***	16.75±1.41 (60.91)***	14.50±2.53(57.43)****

Sample	Inhibition Zone (mm) (% Antibacterial Activity)			
	S. aureus	*E. coli*	*S. typhi*	*B. subtilis*
Test 6				
Control (-)	0.00	0.00	0.00	0.00
Control (+)	25.50± 0.78 (100)	26.75±0.71 (100)	26.75±1.43 (100)	25.25± 1.04 (100)
CA1	18.50±1.41 (60.78)	13.50± 0.12 (50.47)	13.50±0.71 (50.47)	14.50± 0.11 (57.43)
CA3	24.25± 0.71 (95.10)	25.50± 1.21 (95.33)	25.50±1.41 (95.33)	24.50± 0.39 (97.03)
CA5	12.25± 0.13 (48.04)	11.25± 0.23 (42.06)	11.50± 0.10 (42.99)	10.50± 0.07 (41.58)
CC	25.25± 0.71 (99.02)***	18.25±1.12 (68.22)**	18.25±1.41 (68.22)**	17.25± 0.07 (68.32)**
Test 7				
Control (-)	0.00	0.00	0.00	0.00
Control (+)	27.50±1.21 (100)	27.75± 1.32 (100)	25.75±0.71 (100)	25.50±0.89 (100)
CA2	24.50± 0.71(89.09)	25.75±1.51 (92.79)	24.50±1.51 (95.15)	21.50± 0.76 (84.31)
CA4	15.50±0.36 (56.36)	14.25± 0.13 (51.35)	17.50± 0.68 (67.96)	15.50±1.41 (60.78)
CA8	16.25±0.82 (59.09)	15.50± 0.42 (55.86)	17.50± 0.74 (67.96)	18.25± 0.36 (71.57)
CC	23.25±0.71 (84.55)**	27.25±1.41 (98.20)***	23.25± 0.71 (90.29)**	23.25± 0.87 (91.18)***
Test 8				
Control (-)	0.00	0.00	0.00	0.00
Control (+)	27.75±1.45 (100)	26.50± 2.03 (100)	26.75±2.20 (100)	25.25± 0.87 (100)
CA2	25.75 ±1.02 (92.79)	25.50± 0.74 (96.23)	25.25±1.22 (94.39)	20.25± 0.71 (80.20)
CA7	19.25±0.73 (69.37)	24.25± 0.65 (91.51)	25.50±1.31 (95.33)	22.25± 1.41 (88.12)
CA10	22.25±0.41 (80.18)	25.75± 1.01 (97.17)	24.25±2.12 (90.65)	18.75± 1.02 (74.26)
CC	27.25±1.41 (98.20)***	24.75± 0.71 (93.40)**	24.50±0.71 (91.59)**	24.75± 0.00 (98.02)***
Test 9				
Control (-)	0.00	0.00	0.00	0.00
Control (+)	25.75±0.86 (100)	26.75±2.12 (100)	24.50± 0.91 (100)	25.25±1.40 (100)
CA6	14.25±0.71 (55.34)	12.75±2.12 (47.66)	16.50± 0.71 (67.35)	14.25±0.61 (56.44)
CA9	15.25±1.41 (59.22)	15.25± 0.20 (57.01)	16.25± 1.04 (66.33)	17.25±1.12 (68.32)
CA10	25.50±1.13 (99.03)	26.25±2.12 (98.13)	24.50± 0.71 (100)	21.50±1.51 (85.15)
CC	20.50±0.42 (79.61)**	20.25±1.41 (75.70)**	19.25± 0.30 (78.57)**	20.50±1.56 (81.19)**

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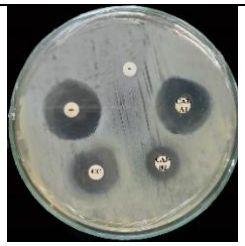
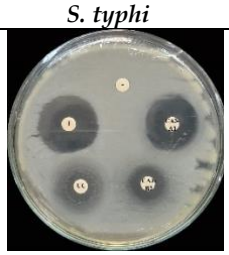
Note: * indifference ** additive *** synergism **** antagonism

In this research, the combination of endophytic fungal extracts isolated from *Physalis angulata* roots revealed that the highest antioxidant agent was observed in test 3, where the combination product achieved a % inhibition value greater than 90%, which is categorized as an indifference effect. This best combination product was derived from the blend of two individual extracts, CA4 and CA6, which each had a % inhibition value > 90%. However, the use of combination products in drug preparations will be more efficient than the use of individual extracts. This is due to the ability of drug combinations to prevent resistance, as they contain several active components with varied structures and functional groups. Additionally, drug combinations can reduce the required dose, thus minimizing adverse/toxic side effects. The blend of two or more different components in a mixture can enhance or complement each other's activity and reflect a broad spectrum of activity [37,38].

The study on the combination effects of endophytic fungal extracts from *Physalis angulata* roots on antibacterial activity showed varying categories. The best combination products with % antibacterial activity ≥ 95% for all test bacteria were combination products from test 2 and test 8. The combination product from test 2 was derived from a blend of two individual extracts, while the combination product from test 8 was derived from a blend of three individual extracts. The combination product of three individual extracts seems to be more efficient, as the dose per individual extract is smaller, thus reducing toxic effects and having a broader spectrum for treating infectious diseases [32,33].

Many factors influence the combination effects of extracts. Studies have shown that the antibacterial effectiveness of plant compounds is affected by various factors: the characteristics of the target microorganism (such as type, genus,species, and strain), the attributes of the plant material [including its botanical source, composition of bioactive compounds, and factors like harvest time, developmental stage, or extraction method], and the chemicalproperties (such as hydrophilicity, lipophilicity, concentration, and pH value) [39]. The findings in this study can be used as a reference for combining extracts with antibiotics to overcome bacterial resistance. Research shows that the antibacterial effects of herbal medicines on clinical isolates indicate that crude extracts serve as sources of resistance-modifying factors [40–42].

Table 8. Antibacterial activity of individual compounds and their combined effects of endophytic fungus *Trichoderma virens* at a concentration of 400 µg/dish

Sample	Inhibition zone (mm)			
	S. aureus	***E. coli***	***S. typhi***	***B. subtilis***
				
Control (-)	0.00	0.00	0.00	0.00
Control (+)	26.10±1.42 (100)	25.30±1.31 (100)	27.03±2.21 (100)	25.51±2.01 (100)
Compound 1	22.05± 0.91 (84.5)	21.02±1.12 (83.1)	23.22±0.81 (85.9)	25.03±1.31 (98.1)
Compound 2	14.23±0.96 (54.5)	14.54±0.71 (57.5)	18.40±0.43 (68.1)	17.81±0.71 (69.8)
Combination	22.31±1.10 (85.5)***	18.61±1.01 (73.6)**	20.24±1.12 (74.9)**	20.53±0.63 (80.5)**

Note: * indifference ** additive *** synergism **** antagonism

The bioactivity properties of endophytic fungal extracts from *Physalis angulata* roots are due to their secondary metabolites. Two compounds were isolated from the endophytic fungus *Trichoderma virens* (CA3), namely 10-hydroxy-benzoisochromen-1-one (1) and 7-hydroxy-benzochromen-6-one (2). The antioxidant activity test results for individual compounds and their combination products showed lower activity compared to individual extracts or their combination products. The decrease in antioxidant activity of pure compounds compared to their extracts may be due to the synergistic effects of more components in the extract, making it more efficient compared to the combination of two pure compounds. Thus, for the development of endophytic fungal as a source of medicinal substances, extract combinations are more efficient than pure compound combinations.

The antibacterial activity of individual compounds and combinations (1:1) from the endophytic fungus *Trichoderma virens* (Figure 2) shows that compound 1 has strong antibacterial activity, with % antibacterial activity ranging from 83.1% to 98.1% against all test bacteria, exceeding the antioxidant activity of the individual extract (CA3), which ranges from 73.91% to 96.15% against all test bacteria. Compound 2 has moderate antibacterial activity, with % antibacterial activity ranging from 54.5% to 69.8%. The combination products are categorized as synergistic and additive, with strong antibacterial activity ranging from 74.9% to 85.5% against all test bacteria. However, the combination products still have lower antibacterial activity compared to the individual compound (compound 2) and the individual extract (CA3). The decrease in antibacterial activity of the combination product may be due to the synergistic effects of more components in the extract, making it more efficient compared to the combination of two pure compounds. These compounds exhibit various structural forms, including differences in the presence, number, and position of hydroxyl groups, as well as the length of the saturated side chain, which contribute to their antibacterial properties. This is linked to the ability of phenolic acids to inhibit ribonucleotide reductase, an enzyme essential for DNA synthesis, thereby preventing bacterial DNA replication. Citric acid's antibacterial effects are due to its physical and chemical properties, which decrease extracellular aggregate production and reduce the hydrophobicity of bacterial cell walls. Antibacterial compounds can destroy bacteria by targeting the cell wall, plasma membrane, protein synthesis, or nucleic acid metabolism. Thus, this combination formula is expected to help avoid unwanted side effects due to higher doses of single ingredients.

Molecular identification results show that the CA3 isolate belongs to the *Trichoderma virens* group. The Trichoderma species have been explored for decades as effective biocontrol agents against many pathogens [43–45]. This capability is certainly related to the secondary metabolites produced by the fungus. *Trichoderma virens* is known as a fungus that can fight phytopathogenic fungi due to the presence of trichocaranes compounds [46–50]. However, some studies reveal that *T. virens* living endophytically in medicinal plants can produce compounds with good bioactivity, making the products from this fungus a potential source of new drugs [51,52]. Literature studies show that 10-hydroxy-benzoisochromen-1-one (1) and 7-hydroxy-benzochromen-6-one (2) compounds have not been found in the endophytic fungus *Trichoderma virens* living on other host plants. These compounds have also not been found in *Physalis angulata*. The active compounds contained in *Physalis angulata* include steroids, alkaloids, flavonoids,terpenoids, saponins, glycosides, carotenoids, tannins, physalins, and withanolides, especially the C28 steroidal lactone series [22-25]. Some studies indicate that some of the bioactive metabolites obtained from endophytic fungi have chemical structures similar or identical to compounds produced by their host plants, while others are different compounds. This presents a great potential of this microbial group for drug discovery [26,53].

Progress in studying the synergistic effects of medicinal plants can be extended to the synergistic interactions of endophytic fungi. With further research into these effects, the potential to develop novel antibacterial medicines derived from endophytic fungi for various treatments, including infection management, may increase. However, the mechanisms underlying these synergistic effects remain largely unknown. A thorough understanding of these mechanisms is necessary to produce standardized and effective therapies. Additionally, evaluating their activity, toxicity, and in vivo bioavailability will determine their potential relevance for future drug development.

4. CONCLUSION

Molecular identification of selected endophytic fungi and phylogenetic analysis showed high similarity with *Trichoderma virens*. Spectroscopic analysis showed that two compounds produced by *T. virens* were 10-hydroxy-benzoisochromene-1-one (1) and 7-hydroxy-benzochromene-6-one (2). The best combination effect with strong antioxidant activity was found in the mixture of CA4+CA6 (test 3), but at a high concentration of 500 µg/mL. The antioxidant activity of compounds 1, 2, and their combination products showed weak antioxidant activity. The highest antibacterial activity (≥ 95%) for the combination product was obtained from the synergistic effect (27.8%) and additive effect (13.9%). Compound 1 had strong antibacterial activity compared to compound 2 and its combination product. Thus, for the development of extracts and pure compounds produced by *Trichoderma virens* as a source of antioxidant compounds showed low potential. This potential can be increased by modifying the structure of compounds 1 and 2 by adding hydroxyl groups to form catechol units on ring A. Meanwhile, the development of extracts and pure compounds produced by *Trichoderma virens* fungi as a source of antibacterial compounds shows great potential. The development of extract combinations is more efficient than pure compound combinations.

5. MATERIALS AND METHODS

5.1 Research Materials and Instruments

Endophytic fungal isolates (CA1-CA10) were taken from stock cultures isolated from *Physalis angulata* roots in 2023 and stored in the Microbiology Laboratory of Sriwijaya University. The materials used in this study include Potato Dextrose Agar, Potato Dextrose Broth, Muller Hinton Agar from Oxoid, 70% alcohol, physiological NaCl solution, NaOCl solution from Onemed, n-hexane, ethylacetate, technical grade methanol distilled before used, aquabidest, DPPH (2,2-diphenyl-1-picrylhydrazyl), ascorbicacid, DMSO, paper disks, tetracycline, *Bacillus subtilis*, *Escherichia coli*, *Salmonella typhi*, and *Staphylococcus aureus*. Microscopic characteristics were analyzed using a digital microscope (Hirox RH-2000). Antioxidant activity was tested using a UV-Vis spectrophotometer (Shimadzu UV-1900). NMR spectroscopy measurements were performed using Bruker H-NMR 700 MHz and C-NMR 176 MHz.

5.2 Rejuvenation of Endophytic Fungi and Morphological Identification

Endophytic fungal isolates were rejuvenated by re-growing the purified isolates in PDA media and incubating them for 5-7 days at room condition. The rejuvenated isolates were then morphologically identified, including macroscopic and microscopic characteristics. The obtained characteristics were compared with the characteristics of the isolates tested in 2023.

5.3 Cultivation and Extraction of Endophytic Fungi

All endophytic fungal isolates were grown in Potato Dextrose Broth (PDB) medium. Mycelia from endophytic fungi on PDA plates were scraped using a cork borer (5x5 mm in diameter) and inoculated into bottles containing 300 mL of PDB medium. Each fungal strain was cultured in 10 bottles. The cultures were incubated for 4 weeks at room temperature under static conditions. After the incubation period, the liquid cultures were extracted with ethyl acetate and evaporated. The liquid broth containing secondary metabolites is partitioned in ethylacetate (ratio 1:1) for 24 hour with three repetitions. All extract are combined and evaporated to provide a concentrated extract of ethylacetate used rotary evaporator (Buchi R300+V300 with interface I300 Pro-F305). Ethyl acetate is a semi-polar solvent. Several studies have revealed that semi-polar solvents are able to bind polar and non-polar compounds so that more complex compounds can be obtained. All concentrated fungal extracts were then tested for antioxidant and antibacterial activities [54].

5.4 Antioxidant Activity Test

DPPH solution was prepared at a concentration of 0.05 mM in methanol. A stock solution was made by dissolving the sample in dimethylsulfoxide (DMSO) at a concentration of 1000 µg/mL. Serial dilutions were then prepared from this stock solution to achieve concentrations of 500, 250, 125, 62.6, 31.25, 15.62, and 7.81 µg/mL. To 0.2 mL of each sample solution, 3.8 mL of the 0.05 mM DPPH solution was added. The mixture was homogenized and left to sit in the dark for 30 minute. Absorbance was then measured by using a UV-Vis spectrophotometer at a wavelength of 517 nm. Ascorbic acid, treated in the same manner as the sample, was used as the standard antioxidant. The antioxidant activity of the sample was assessed by determining the percentage inhibition of DPPH absorption, calculated using the corresponding formula [55]-[58]:

$$\% \text{ inhibition} = \frac{\text{control absorbance} - \text{sample absorbance}}{\text{control absorbance}} \text{x } 100\ \%$$

5.5 Antibacterial Activity Test

The antibacterial activity of the extracts was assessed using the agar disk diffusion method against four bacterial strains: *Salmonella typhi, Escherichia coli, Staphylococcus aureus,* and *Bacillus subtilis*. Bacterial suspensions were adjusted to a concentration of 1.5 x 10^8 CFU/mL, following the 0.5 McFarland standard. A 100µL aliquot of this suspension, diluted to 5 x 10^5 CFU/mL, was evenly spread onto petri dishes containing 20mL of Mueller Hinton Agar (MHA). Paper disks (6 mm) infused with 10 µL of concentrated extract in 10% DMSO (400 µg/disk) were then placed on the agar plates. As controls, 10% DMSO was used as the negative control, and tetracycline (30 µg/disk) was used as the positive control. After incubating the plates at 37°C for 24 hours, the zones of inhibition around the disks were measured and compared to the standard antibiotic to evaluate the antibacterial activity percentage [55]-[58]:

Strong: $\frac{A}{B}$x 100% > 70 %; Moderate: 50 % <$\frac{A}{B}$x 100%< 70 %; Weak: $\frac{A}{B}$x 100% <50 %

A: Sample

B: Positive control

5.6 Isolation and Identification of Compounds

Active extracts were analyzed using Thin LayerChromatography (TLC) with different eluent systems to assess the secondary metabolite content and identify the optimal eluent for preliminary separation. The initial fractionation was then carried out using Gravity Column Chromatography (CC). The separated fractions were dissolved in asuitable solvent and impregnated with silicagel 60 (70-230 mesh) in a 1:2 ratio of sample weight to impregnated silica. A 1:10 ratio of sample weight to silica gel was used for the elution process, which employed the eluent identified from TLC analysis. The fractions were collected in bottles and analyzed by TLC. Eluates with similar spot patterns were combined into a single fraction and evaporated again using a rotary evaporator. Fractions showing well-separated spot patterns were further purified by re-CC, recrystallization, or washing to isolate pure compounds.

5.7 Molecular Identification of Endophytic Fungi

DNA from endophytic fungi was extracted from 7-day-old mycelium grown on the surface of PDA media using a Promega kit. Molecular identification was conducted based on the Internal Transcribed Spacer (ITS) rDNA region. Universal primersITS1(5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were used for amplification. The forward and reverse primer sequences were compiled using the Bioedit program to remove unnecessary sequences. The assembled sequence was then submitted to the BLAST at http://blast.ncbi.nlm.nih.gov/Blast.cgi. A phylogenetic tree was constructed using the Neighbor-joining method, with a bootstrap value set at 1000 for reliability [59].

5.8 Combination Effects of Extract-Extract and Compound-Compound

5.8.1 Effects of Combination on Antioxidant Activity

Each endophytic fungal extract (CA1-CA10) and pure compound (C1-C2) was prepared at a concentration of 500 µg/mL in a total volume of 5 mL. Combination tests were conducted by mixing two or three components in a 1:1 ratio. A total of nine extract combinations were prepared, designated as tests 1-9. The antioxidant activity of the individual components and their combinations was measured for each test. The % inhibition was calculated, and the color change was observed. The more yellow the test solution, the more active the antioxidant [60].

5.8.2 Effects of Combination on Antibacterial Activity

Each endophytic fungal extract (CA1-CA10) and pure compound (C1-C2) was prepared at a concentration of 4% (400 µg/dish) in 1 mL of 10% DMSO, with tetracycline (30 µg/dish) serving as the positive control. For combination tests, two or three extract components were mixed in a 1:1 ratio. Nine different extract combinations were created, labeled as tests 1-9. The antibacterial activity was evaluated using the KirbyBauer method, with each test performed in duplicate. The inhibition zone diameter was recorded for each test, and the % antibacterial activity was calculated accordingly.

5.8.3 *Analysis of Combination Effects*

Combination effects were classified into four categories: indifference, additive, synergism, and antagonism [61].
Indifference: activity of CP = activity of the most active IC
Additive: activity of CP = sum of activities of each IC
Synergism: activity of CP > activity of the most active IC
Antagonism: activity of CP < activity of the least active IC CP = combination products, IC = individual components

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