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Original Research Article

Antioxidant Activity of Endophytic Fungi Isolated from Cashew (*Anacardium occidentale*) Leaves

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ABSTRACT

Cashew (*Anacardium occidentale*) is a plant used by Indonesian people a traditional medicine. The leaves are consumed as fresh vegetables and to treat various diseases. Endophytic fungi can live symbiotically in plant tissue and have the ability to produce substances called secondary metabolites. The research aims to isolate endophytic fungi from *cashew* leaves and identify them morphologically and molecularly as well as identify the secondary metabolite from the most active antioxidant extract. The identification of endophytic fungal species was done using morphological and molecular techniques. Both the culture and extraction processes used Potato Dextrose Broth (PDB) media and ethyl acetate as a solvent. The ethyl acetate extract, which is included in the strong antioxidant category, was continued to the stage of isolating the pure compound and then its chemical structure was analyzed using spectroscopic methods. A total of eight endophytic fungus (RDM1-RDM8) were found on *cashew* leaves. Ethyl acetate extract from the endophytic fungi RDM4 has the strongest antioxidant activity with an IC_{50}= 13.80 µg/mL. Morphological and molecular identification through phylogenetic analysis shows that RDM4 is *Neopestalotiopsis clavispora*. This endophytic fungus produces 5-isopropyl-4-methylfuran-2-one as a secondary metabolite. Ethyl acetate extract of the *Neopestalotiopsis clavispora* has more potential to be developed as a new source of antioxidants than the pure compound because it is thought that there is a synergistic effect between the compound components contained in the extract.

Keywords: Antioxidant Activity, *Cashew*, Endophytic Fungi, *Neopestalotipsis clavispora*, Secondary Metabolite.

Introduction

Oxidative stress (OS), also known as free radical imbalance, is a condition when the body's production of free radicals and antioxidant defenses are out of balance.[1–3] Chronic conditions include cardiovascular disease, diabetes, cataracts, neurological conditions, Alzheimer's disease, cancer, atherosclerosis, bronchial asthma, and rheumatoid arthritis are all strongly linked to oxidative stress.[4–6] Antioxidants work by delaying or preventing the oxidation of other substances, blocking oxidation reactions by scavenging free radicals, and reducing the amount of oxidative damage to biological processes. ROS/RNS.[7–9] Because of their capacity to lower oxidative stress, antioxidants are crucial for maintaining human health as well as for preventing and treating illness.[10,11] The prevention and treatment of neurodegenerative illnesses can benefit from antioxidants.[12–14]

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An alternative natural antioxidant is required since the usage of synthetic antioxidants to stop free radical damage can have severe side effects.[15,16]

Natural antioxidant compounds can be obtained from the *cashew* leaves (Anacardium occidentale L). *Cashew* tree parts can be utilized to combat free radicals and cure infectious diseases brought on by bacteria and fungus.[17–19] The compounds contained in *cashew* leaf extract have been reported to contain ascorbic acid, quercetin, quercetin 3-o-rutinoside, quercetin 3-o-rhamnoside, zoapatanolida A, agathisflavone, anacardisin, pentagalloylglucose, methyl gallate, flavonoids, tannins, triterpenes, saponins, alkaloids. These ingredients have been proven as antioxidants and anticancer.[20–23] It is difficult to extract traditional medicines from plants due to the poor yields of bioactive chemicals caused by the complexity of the metabolites found in medicinal plants. Therefore, endophytic fungi can be used as an alternative source to produce bioactive compounds.

History demonstrates that endophytic fungi's bioactive metabolite molecules can aid in the development of novel therapeutics. The "frontline" antibiotic penicillin, which is made by *Penicillium chrysogenum*, was discovered; this discovery marked a significant turning point in the development of antibiotics and saved millions of lives.[24] The endophytic fungus *Taxomyces andreanae* from the *Taxus brevifolia* plant, which is capable of producing the taxol molecule, is the source of another bioactive substance. The medication taxol is authorized for the management of advanced ovarian, lung, and breast cancers. First obtained from *Taxus brevifolia* and other uncommon and

slow-growing plants in the Taxus family, this remarkable anticancer medication also produces trace quantities of taxol. [25,26]

One of the most important sources for the development of new pharmaceuticals is natural compounds obtained from endophytic fungi.[27–31] According to several research, the majority of secondary metabolites derived from endophytic fungus have a particular chemical structure, either through the creation of new molecules that are distinct from their hosts or secondary metabolites that are similar to their hosts' secondary metabolites.[29,32–36] The culinary, agricultural, and pharmaceutical industries may all benefit from the abundance of beneficial secondary metabolites that are produced by endophytic fungus. These compounds have antibacterial, antifungal, , antioxidant, immune-suppressing, antiparasiticanti-inflammatory, antiviral, and anticancer properties. Peptides, alkaloids, terpenoids, steroids, lactones, phenylpropanoids, quinones, anthraquinones, flavonoids, phenols, xanthones, indole derivatives, and phenolic acids are some of these.[37–40] *Neopestalotiopsis clavispora* is one of the various types of endophytic fungi that are reported to have various secondary metabolites.[41,42]

The endophytic fungus *N. clavispora* is a common pathogenic fungus found infecting plants.[43–46] According to studies, this fungus causes post-harvest rot, canker scab, stem rot, leaf blight, leaf spot, pod rot, and leaf blight.[47–49] However, there are studies which reveal that *N. clavispora* is endophytic and is found in various plant tissues, ranging from leaves, stems, to roots.[50,51] The content of secondary metabolites of this fungus is also diverse, such as polyketide and terpenoid groups, in which these compounds have very high bioactivity.[52,53] The bioactivity and secondary metabolites of the endophytic fungus *N. clavispora* are still very limited, and further research is being done to produce or demonstrate antioxidant compounds from endophytic fungal isolated from *A. occidentale* leaves. Based on the aforementioned literature studies, the benefits of this fungus are still unknown.

Materials and Methods

Preparation of Plant Samples

The leaves of *A. occidentale* were collected fresh and healthy on 16 July 2023 from Sriwijaya University, Ogan Ilir, South Sumatra, Indonesia. It was identified in Yayasan Generasi Biologi Indonesia, Gresik with letter number: 08.115/Genbinesia/IX/2023.

Sample Sterilization and Isolation of Endophytic Fungi

For around five minutes, the leaves of *A. occidentale* were cleaned thoroughly under running water. By submerging the surface of the samples in 70% alcohol for about a minute and washing it with sterile distilled water for about a minute, you may sterilize it. The sample was then soaked with sodium hypochloride (NaOCl) for ± 30 seconds, rinsed again with 70% etanol for ± 30 seconds, and rinsed with distilled water for ± 1 minute. Sterile samples were crushed aseptically using a mortar and pestle in Laminar Air Flow. Samples were deeply inoculated in potato dextrose agar media in a petri dish, incubated at room temperature for 3-7 days. Until there were discernible fungi growing, observations were done every day. Then, fungus colonies on PDA media with various morphological traits (shape, color, and size) were purified. Colonies were purified by moving them to fresh PDA media and incubating them there for two consecutive days at room temperature. The transfer of pure fungal colonies to culture media allowed for the investigation of macroscopic and microscopic characteristics.[58]

Characterization and Identification of Endophytic Fungi

The color, texture, exudate droplet presence, radial lines, and concentric circles of the endophytic fungal colonies were assessed when they were between three and seven days old. The Henrici's slide culture method was used to prepare microscope preparations in order to study microscopic properties. The spores' morphology and the existence or lack of divisions on the hyphae are examples of microscopic observations. Identification was done using newly discovered macroscopic and microscopic traits in comparison to existing literature.[54-56]

Cultivation and Extraction of Endophytic Fungi

In 3 bottles of culture media, which contained 300 ml of Potato Dextrose Agar (PDB) and 5 blocks (5 mm in diameter) of pure culture agar, the endophytic fungus that was isolated from the *cashew* leaves were cultivated. For 30 days, the cultures were cultured at room temperature in a fixed environment. Filter paper was employed to remove the mycelia from the medium following the incubation period. In addition, the medium was extracted after being diluted with ethyl acetate (1:1). A rotary evaporator was used to concentrate the extract. The extract is then concentrated again until dry in a 45 °C oven. The concentrated extract is then weighed.[56,57]

Antioxidant Activity Test

The DPPH technique for measuring antioxidant activity. Every endophytic fungal extract was dissolved in methanol three times at doses of 1000, 500, 250, 125, 62.5, 31, 25, and 15.625 µg/mL. A 3.8 mL solution of 0.5 mM DPPH was added to 0.2 mL of each concentration. After homogenizing the mixture, it was stored for 30 minutes in a dark tube. Using a UV Vis spectrophotometer, the absorbance value was determined at a maximum of 517 nm.[58,59] Ascorbic acid served as the standard antioxidant in this experiment. Antioxidant activity was estimated using the IC_{50} value and the percentage of DPPH absorption inhibition.[60]

$$\%\ \text{Inhibition} = \frac{A_k - A_s}{A_s}$$

A_k = Absorbance of control
A_s = Absorbance of samples

Molecular Identification of Endophytic Fungi

The most likely endophytic fungi were then identified based on their bioactivity. Internal Transcribed Spacer (ITS) DNA of rDNA serves as the basis for identification, and ITS1 and ITS4 primers were used in the amplification process. Forward and reverse primer DNA sequence assembly was produced with the aid of the Bioedit tool. The sequences are then matched using basic local alignment search tool (BLAST), http://blast.ncbi.nlm.nih.gov/Blast.cgi is the website address for it. Following the CLUSTAL W method of the MEGA11 program's alignment of the sample sequences and databases, The Neighbor-joining tree technique was used to create phylogenetic trees, with a bootstrap value of 1000.[61]

Isolation and Identification of Secondary Metabolites

Using silica gel G60 (70-230 mesh) in a 1:1 ratio, up to 2 g of the endophytic fungus RDM4's ethyl acetate extract was preabsorbed. Then separated by gravity column chromatography (CC) method with a gradient eluent system, namely n-hexane 100% (100 mL), n-hexane-ethyl acetate (9:1 = 100 mL), n-hexane-ethyl acetate (7:3 = 100 mL), n-hexane-ethyl acetate (5:5 = 100 mL), n-hexane-ethyl acetate (3:7 = 100 mL), e 100% ethyl acetate (100 mL) mL), ethyl acetate-methanol (9.5:0.5 = 100 mL), ethyl acetate-methanol (9:1 = 100 mL), 100% methanol (30 mL). Silica gel 60 G (70-230 mesh) was the stationary phase that was employed. A vial was used to collect the eluate every 10 mL. Thin layer chromatography (TLC) was used to evaluate the eluate, wherein identical stain patterns were merged into one fraction to produce many column fractions. To produce a pure chemical, the column fraction was purified using chromatographic methods and washed with the proper solvent mixture. The identification of chemical structures was done using spectroscopic techniques such ^{1}H-NMR, ^{13}C-NMR, HMQC, and HMBC.

Results and Discussion

Isolation and Identification of Cashew Leaf Endophytic Fungi
Morphological Identification

The results of the isolation of endophytic fungus on *cashew* leaves found 8 isolates (codes RDM1 to RDM8). Colonies of 8 endophytic fungal isolates isolated from *cashew (Anacardium occidentale*) leaves showed diverse macroscopic characteristics including shape and color and distinctive microscopic characters (Figure 1 and Figure 2). The color of the colonies that appear on *cashew* leaves is dominated by

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white, pink, gray and black. The results of macroscopic and microscopic observations of the characteristics of endophytic fungal isolates can be seen in Table 1 and Table 2.

Eight genera and eight species of endophytic fungi found on *A. occidentale* leaves have been successfully isolated, which were then identified based on morphological characters including macroscopic and microscopic characters, namely *Humicola tainanensis, Microsporum nanum, Chaetomium sp., Neopestalotiopsis clavispora, Rhizoctonia solani, Chrysosporium sp., Verticilium sp.*, and *Phytopthora sp*.

Antioxidant Activity of Endophytic Fungus Extract from Cashew Leaves

Endophytic fungus from *Anacardium occidentale* leaves extracted using ethyl acetate has potential as antioxidants (Table 3). The extracts tested showed antioxidant activity in the categories very strong, strong, medium and weak.

Endophytic fungal extracts showed varying antioxidant activity, ranging from very strong, strong, moderate, and weak. However, when compared with the IC_{50} value of ascorbic acid (10,083 µg/mL; very strong), the IC_{50} value of endophytic fungal extract is still lower. However, the IC_{50} value of ethyl acetate extract from the endophytic fungus isolate RDM4 (13.80 µg/mL; very strong) was closest to the IC_{50} value of ascorbic acid. Based on its morphological characters, RDM4 was identified as *Neopestalotiopsis clavispora*. Table 3 also displays the outcomes of studies to determine the antioxidant activity of chemicals derived from the most active endophytic fungus. The findings demonstrated that the compounds have ineffective antioxidant action (IC50 > 100 µg/mL).

Molecular Identification

A molecular comparison of the RDM4 isolate and *Neopestalotiopsis clavispora* revealed 100% identity. Figure 3 displays the evolutionary tree, which has the following order:

GTAACAAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTA
TAGAGTTTTCTAAACTCCCAACCCATGTGAACTTACCTTTTG
TTGCCTCGGCAGAAGTTATAGGTCTTCTTATAGCTGCTGCCG
GTGGACCATTAAACTCTTGTTATTTTATGTAATCTGAGCGTC
TTATTTTAATAAGTCAAAACTTTCAACAACGGATCTCTTGGT
TCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAAT
GTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGC
ACATTGCGCCCATTAGTATTCTAGTGGGCATGCCTGTTCGAG
CGTCATTTCAACCCTTAAGCCTAGCTTAGTGTTGGGAATCTA
CTTCTCTTAGGAGTTGTAGTTCCTGAAATACAACGGCGGATT
TGTAGTATCCTCTGAGCGTAGTAATTTTTTTCTCGCTTTTGTT
AGGTGCTATAACTCCCAGCCGCTAAACCCCC.

Compound Isolation and Identification

Column chromatography (CC) was used to separate the RDM4 ethyl acetate extract (2 g) using an eluent with increasing polarity. To monitor the column chromatography eluate, thin layer chromatography (TLC) was used, which was stored in 75 vials (10 mL each), using n-hexane and ethyl acetate as the mobile phases (5:5). Four column fractions, designated F1 through F4, were created by combining the TLC profiles with identical chromatogram patterns into a single fraction. The n-hexane:EtOAc (2:8) solvent combination was used to rinse the F3 fraction in order to purify it, yielding compound 1 in the form of yellow crystals (30.2 mg).

Figure 1: Morphological characteristics of endophytic fungal colonies from *Anacardium occidentale* leaves. Macroscopic characters (front view (a); reverse view (b))

Table 1: Characteristics of endophytic fungal colonies from *Anacardium occidentale* leaves

Code	Surface Colony	Reverse Colony	Structure	Elevation	Pattern	Exudate Drops	Radial line	Concentric circle
RDM 1	Dark gray	Dark grey to black	Cottony	Umbonate	Zonate	-	-	-
RDM 2	White	Beige	Powder	Umbonate	Radiate	-	-	√
RDM 3	Grayish	Tan gray	Cottony	Rugose	Zonate	-	√	√
RDM 4	Pink to violet	Pink to violet	Cottony	Rugose	Radiate	-	√	√
RDM 5	Grayish	Gray to brown	Cottony	Rugose	Zonate	-	√	√
RDM 6	Yellowish brown	Yellowish brown	Velvety	Rugose	Radiate	-	√	√
RDM 7	Pink	Pink	Velvety	Rugose	Zonate	-	√	-
RDM 8	Grayish to black	Black	Cottony	Umbonate	Zonate	-	-	-

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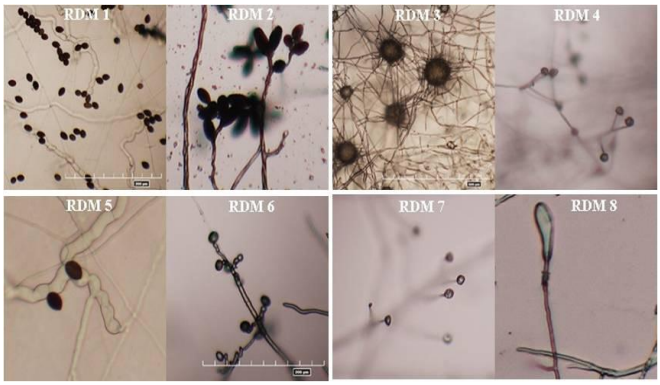

Figure 2: Microscopic characteristics of endophytic fungi from *Anacardium occidentale* leaves

The ^{1}H-NMR spectrum (500 MHz, CDCl3) (Figure 4) showed six proton signals consisting of three methyl proton signals and three methine proton signals. In the spectrum, it can be seen that there are two methyl protons with doublet multiplicity and the same plot constant, namely at δ_H 1.17 (3H, d, J=6.5) and 1.31 ppm (3H, d, J=6.5). This indicates that the two methyl groups are attached to the same methine carbon atom to form an isopropyl group. Another methyl proton signal with singlet multiplicity appears at δ_H 2.03 (3H, s). This indicates that compound 1 has a methyl group attached to the quaternary sp2 carbon atom. Furthermore, there are three widened methine signals caused by the long range capling between the protons of the methine group and the 4 bond distance protons. The three methine protons consist of a vinylic sp2 proton, an oxymethane proton and an isopropyl methine proton.

The ^{13}C-NMR spectrum (Fig. 5A) showed 8 carbon signals composed three sp2 carbon signals and five sp3 carbon signals respectively. This indicates that compound 1 is not an aromatic compound. The ^{13}C-NMR spectrum data amplified by the HMQC spectrum (Fig. 5B) shows that there is an ester carbonyl group at δ_C 160.2 ppm, a vinylic carbon at δ_C 95.7 ppm, sp2 quaternary carbon at δC 147.7 ppm, an oxymethine carbon at δ_C 70.2 ppm, a sp3 methine carbon at δ_C 37.8 ppm and three methyl carbons at δ_C 20.3; 19.1; and 9.1 ppm.

The HMBC spectrum (Figure 6) shows the correlation of the methyl proton at δ_H 2.03 ppm with the sp2 quaternary carbon (δ_C 147.7 ppm) and the cyclic ester carbonyl carbon (δ_C 160.2 ppm). This indicates that the methyl proton is bound to the unsaturated carbon α, β carbonyl. Furthermore, there is a correlation between methyl protons at δ_H 1.31 ppm with sp3 methine carbon (δ_C 37.8 ppm) and oxymethine carbon (δ_C 70.2 ppm). The last methyl proton, at δ_H 1.17 ppm, correlates with sp3 methine carbon (δ_C 37.8 ppm), oxymethine carbon (δ_C 70.2 ppm), and unsaturated carbon *α, β* carbonyl (δ_C 147.7 ppm). This indicates that the two methyl groups are attached to the same methine carbon atom (δ_C 37.8 ppm) in the form of an isopropyl group attached to the oxymethine carbon atom (δ_C 70.2 ppm). The long-distance correlation between the methyl proton (δ_H 1.17 ppm) and the unsaturated carbon α, β carbonyl (δ_C 147.7 ppm) may be due to the close stereochemistry. Table 4 displays the NMR spectra data for compound 1.

In the ^{1}H-NMR spectrum, a clean base line without impurities can be seen. The emerging proton signals shows appropriate integration and multiplicity. However, in the ^{13}C-NMR spectrum there are low signals on the base line. This may be because there is a difference in time when measuring the spectrum, which has an impact on the purity of the compound. However, these signals were only identified as impurities because they did not have a significant correlation in the HMQC and HMBC spectra.

Compound 1 is a five-ring unsaturated cyclic ester compound with the molecular formula $C_8H_{12}O_2$, Double Bond Equivalent (DBE) is 3, consisting of one for the cyclic, one for the carbonyl group, and one for the carbon-carbon double bond. This information is based on NMR spectroscopic data of compound 1, whose chemical structure is thought to be the compound 5-isopropyl-4-methylfuran-2-one. Using the DPPH test, compound 1 is not effective as an antioxidant. Free radicals in DPPH are extremely reactive and can absorb electrons or other hydrogen radicals to transform into stable diamagnetic molecules.[62]

Table 2: Microscopic characteristics of endophytic fungal colonies from *Anacardium o*ccidentale leaves

Isolate	Spore	Shape	Hyphae	Characteristic	Species of Identification
RDM 1	Hyaline	Subglobose	Septate	Conidiosphores hyaline, erect, simple or branched, granualate, solitary or catenutale on creeping hyphae.	*Humicola tainanensis*
RDM 2	Conidia	Subglobose	Septate	The short conidiosphores, septate hyphae, club shaped and smooth walled, may also be present.	*Microsporum nanum*
RDM 3	Conidia	Ovoid	Septate	Hyphae are septate, round, oval, or flask-shaped perithecia.	*Chaetomium sp.*
RDM 4	Hyaline	Subglobose	Septate	Conidiosphores hyaline, simple or branched, bearing conidia in chains and/or spore masses at the apexes of branches.	*Neopestalotiopsis clavispora*
RDM 5	Conidia	Subglobose	Septate	Hyphae pale brown, branched, with nearly right-angled side branches constriched basally.	*Rhizoctonia solani*
RDM 6	Hyaline	Globose	Septate	Conidiosphores lacking, or very short, simple or branched, hyaline, bearing conidia at apical parts, or rarely directly on hypae	*Chrysosporium sp.*
RDM 7	Conidia	Subglobose	Septate	Conidiosphores erect, hyaline, bearing spore masses at verticilate, alternate, opposite.	*Verticilium sp.*
RDM 8	Sporangia	Subglobose	Septate	Sporangia ellipsodial, zoospores developed inside sporagia.	*Phytopthora sp.*

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Table 3: Antioxidant activity of endophytic fungal extract and its pure compound from *Anacardium o*ccidentale leaves compared with ascorbic acid as antioxidant standard

Isolate code	Species	Ethyl acetate Extract Weight (gram)	Antioxidant activity IC_{50} (µg/mL)
RDM 1	*Humicola tainanensis*	1.1	30.65 ***
RDM 2	*Microsporum nanum*	1.2	84.66 ***
RDM 3	*Chaetomium sp.*	1.0	61.14 ***
RDM 4	*Neopestalotiopsis clavispora*	1.8	13.80 ****
RDM 5	*Rhizoctonia solani*	0.7	14.57 ****
RDM 6	*Chrysosporium sp.*	0.6	25.21 ***
RDM 7	*Verticilium sp.*	1.4	16.85 ****
RDM 8	*Phytopthora sp.*	0.9	119.9 **
Pure Compound			>100 (in active)
Ascorbic Acid			10,083 ****

Note: antioxidant activity IC_{50} (µg/mL): ****very strong < 20 µg/mL ***strong < 100 µg/mL; **moderate 100-500 µg/mL; * weak > 500 µg/mL

Table 4: The NMR data of compound 1 (^{1}H-500 MHz, ^{13}C-125 MHz in CD_3OD)

No. C	δ_C ppm 1	δ_H ppm (ΣH. Multiplicity, Hz) 1	HMBC 1
2	160.2		
3	95.7	5.52	
4	147.7		
5	70.2	3.91	
6	37.8	2.68	
7	20.3	1.31 (3H, d, J=6.5)	70.2; 37.8
8	19.1	1.17 (3H, d, J=6.5)	147.7; 70.2; 37.8
9	9.1	2.03 (3H, s)	147.7; 160.2

Compound 1 lacks a hydroxyl group, which prevents DPPH radicals from causing proton abstraction, which makes it incapable of reducing DPPH free radicals. Consequently, in tests of antioxidant activity, residual DPPH radicals remain strong, yielding a high IC_{50} value (> 100 µg/mL). The IC_{50} value was used to categorise the degree of antioxidant activity. A compound is classified as very active, active, and not active as an antioxidant if it has an IC50 value respectively: < 10; < 100; and > 100 µg/mL.[63] Figure 7 illustrates the molecular structure of compound 1, which includes the location of protons, carbons, and HMBC correlations (A), as well as the numbering of carbon atoms (A).

The genus *Neopestalothiopsis* is known as a pathogenic organism that infects plants. This genus does not have a host species so its ability to infect various types of plants is very high.[64–66] However, several studies have revealed that the *Neopestalotiopsis* genus is endophytic on plants, such as *N. formicarum*, *N. zimbabwana*, and *N. clavispora*.[67,68] This study found endophytic fungi *N. clavispora* from the leaves of *A. occidentale*. Several references reveal that the endophytic fungi *N. clavispora* is found in the roots, stems and leaves. This finding completes the reference that *N. clavispora* is endophytic in all parts of the plant. Fungi that are endophytic in medicinal plants usually have the same bioactivity as their host plants.[69–72] Its ability to produce secondary metabolites is very useful for host plants for defense so that the interaction between endophytic fungus and the host plants is mutually beneficial. This species of fungus has been reported to contain secondary metabolites with good bioactivity.[41,73,74]

N. clavispora's ethyl acetate extract showed extremely potent antioxidant activity (IC_{50} < 20 g/mL). The two predominant secondary metabolites in *N. clavispora* are flavonoids and phenolics.[52,53] Oxidative stress can be decreased by flavonoid and phenolic compounds because they have hydroxyl groups and long saturated side chains, so these compounds are able to scavenge free radicals.[75–81] The secondary metabolite components in endophytic fungal extracts are said to be the same as those in their host plants, according to the literature. This occurs due to mutualistic interactions among endophytic fungal and their host plants.[82–85]

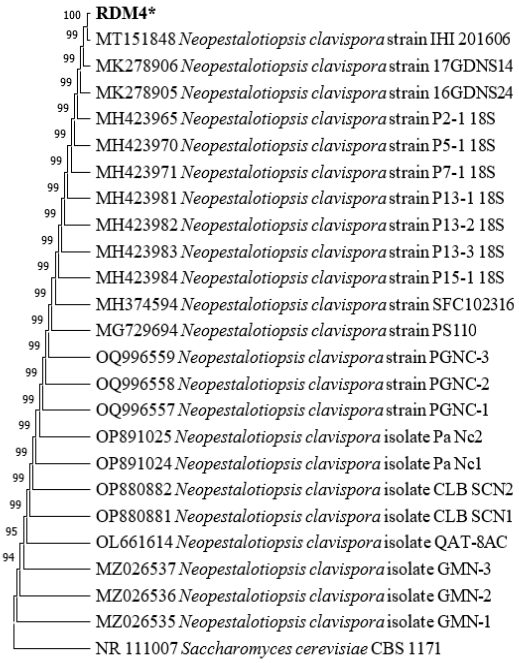

Figure 3: Phylogenetic tree of isolate RDM4* (*Neopestalotiopsis clavispora*)

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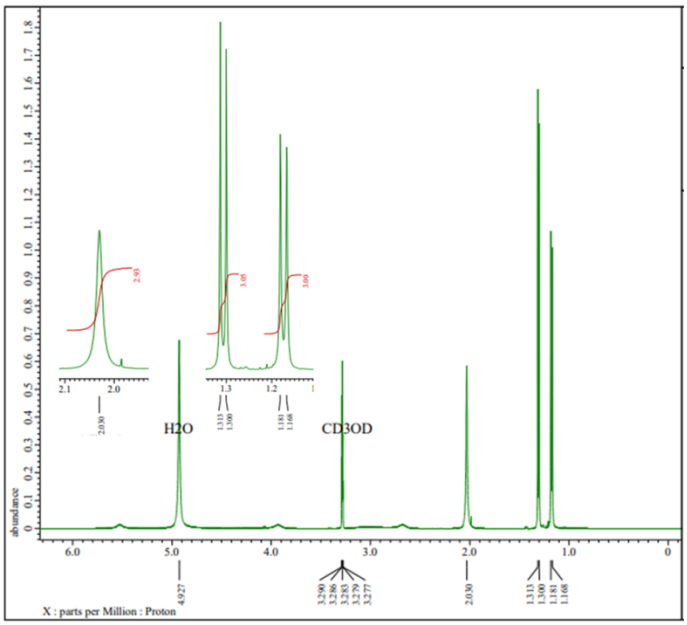

Figure 4: The [1]H-NMR spectral of compound 1

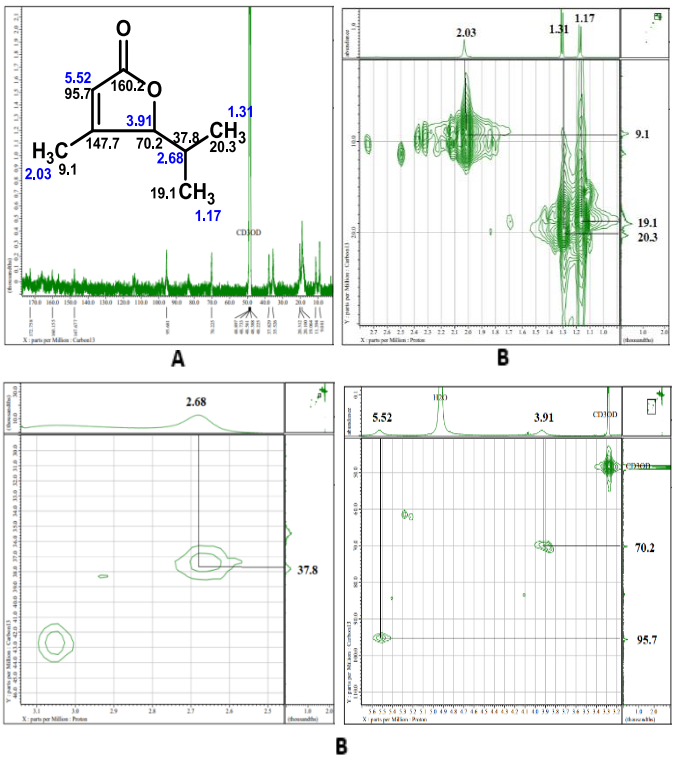

Figure 5: The [13]C-NMR broad band has impurities (A) and HMQC (B) spectral of compound 1.

Compounds produced by *N. clavispora* from *A. occidentale* leaves (Figure 7) showed inactive antioxidant activity (IC_{50} > 100 µg/mL). In this study, no O-H (hydroxyl) bonds were found, but C-H (carbonyl) bonds were found. Generally, antioxidants are molecules containing active hydroxyl groups, such as vitamins E and C, polyphenolic and flavonol compounds, which are strong radical scavengers.[86,87] Compound 1's lack of a hydroxyl group rendered it ineffective as an antioxidant since there were no protons available for free radicals to abstract. According to studies, removing hydroxyl groups can decrease coplanarity, which can decrease a compound's capacity for scavenging free radicals.[76,88–91] Thus, pure antioxidant compound may still be present in the ethyl acetate crude extract of *N. clavispora* which have not been isolated in this study. Another possibility is the occurrence of synergistic between the components contained in the extract. For further development as a drug candidate, it can be used in extract form. If using compound 1 as a drug candidate, it is necessary to modify the structure to increase its antioxidant activity.

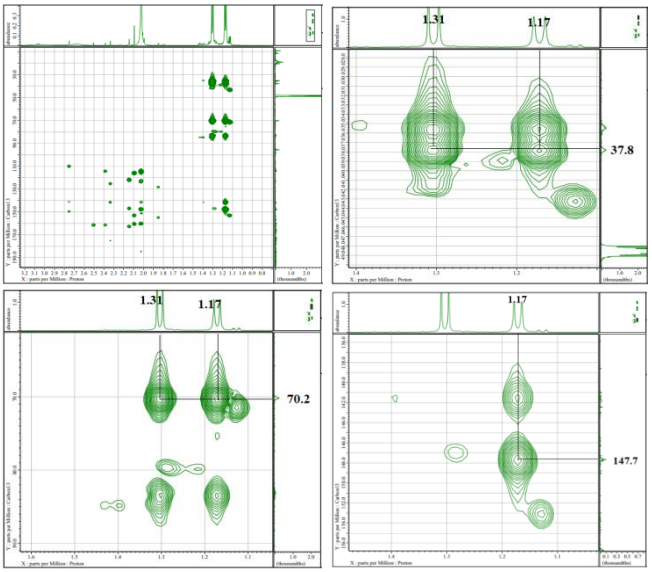

Figure 6: The HMBC spectral of compound 1

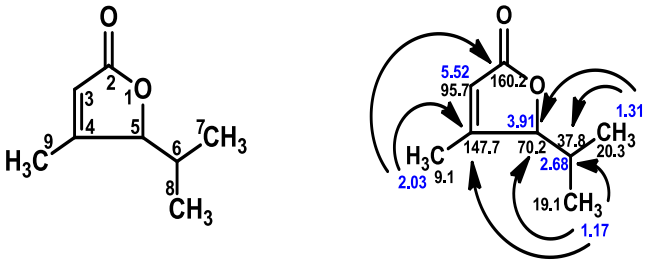

Figure 7: Structure of compound 1: 5-isopropyl-4-methylfuran-2-one with the numbering of carbon atoms (A), the placement of chemical shifts of protons, carbons, and selected HMBC correlations (B).

Conclusion

5-isopropyl-4-methylfuran-2-one produced by the endophytic fungus *Neopestalotiopsis clavispora* is not active as an antioxidant. This is because compound 1 does not have a hydroxyl group so that proton abstraction does not occur by DPPH radicals. This compound comes from selected ethyl acetate extract which provides strong antioxidant activity. It is suspected that compounds that have strong antioxidant activity are still left in the ethyl acetate extract and need to be isolated in further research.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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