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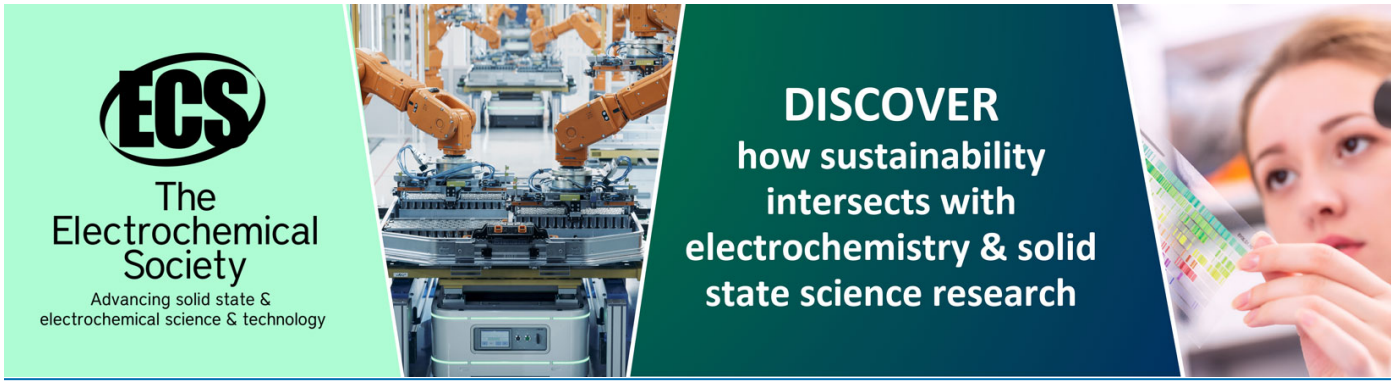

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Determination of structure and anticancer activity of MM_2 compound, isolated from endophytic fungus *Aspergillus terreus*-RTN3 of *Alpinia chinensis* Rosc.

T N M Vo[1*], D N Nguyen[2] and V T Nguyen[1]

[1]Faculty of Pharmacy, Nguyen Tat Thanh University, Ho Chi Minh City 70000, Vietnam
[2]Faculty of Pharmacy, University of Medicine and Pharmacy Ho Chi Minh city 70000, Vietnam

*Corresponding author: vtnmy@ntt.edu.vn, vongocmy1984@gmail.com

Abstract. The study has isolated endogenous *A. terreus*-RTN3 strain from young stems of *Alpinia chinensis* Rosc.-Zingiberaceae family. Cultivating and extracting compounds containing metabolites that are resistant to *Staphylococcus aureus* and Methicillin-resistant *Staphylococcus aureus* (MRSA). Determining the number of antibacterial compounds in the crude extract by thin-layer chromatography and vacuum liquid chromatography (VLC), the research has isolated pure MM_2 compound with high antioxidant activity and spectral resolution to find the structure compound MM_2 (Methyl 2-((4-amino-2-bromo-3-methyl-5-thioxocyclopenta-1,3-dien-1-yl) oxygen) -4-hydroxy-6-methoxybenzoate). This is a new compound that has not been announced in any works in the world. MM2 compound has a high antioxidant activity, the ability to catch α, α-diphenyl-β-picrylhydrazyl (DPPH) free radicals increases linearly by concentration, at concentration 400ppm of MM_2 is capable of catching over 75% of DPPH free radicals. The MM_2 compound also has the ability to inhibit 4 test cell lines: breast cancer (MCF-7), cervical cancer (Hela), liver cancer (Hep G2) and lung cancer (NCI-H460). The results showed that MM_2 compound's ability to inhibit cancer cell lines increased linearly, at concentrations of 100 µg/ml of MM_2 compound, which inhibited nearly 80% of cancer cell lines and was high most in liver cancer cell line HepG2 (at a concentration of 100 µg/ml inhibits 86.82% of liver cancer cells HepG2).

1. Introduction

In plants, endophytic fungi and plant are closely related, endophytic fungi can use substances in plants as nutrients to survive. In return, they bring many benefits to the plant, playing an important role in protecting the host plant against the harmful effects of insects, harmful microorganisms or environmental disadvantages. Recently, secondary fungi metabolites, especially endophytic fungi, are gaining interest because they can produce many bioactive metabolites with antibacterial, anticancer and anti-oxidant properties [3]. Some endophytic fungi are noted as *Aspergillus, Penicllium, Fusarium* due to the production of many metabolites for biological effects such as antibacterial, antiviral, anticancer, etc. in which *A. terreus* spieces product many compounds have properties antibacterial such as terremid A, terremid B, terrein,etc [1]. *A. terreus* - RTN3 has isolated from young stems of *A. chinensis* Rosc.-Zingiberaceae family. In culture media, *A. terreus* - RTN3 is a microbe that is resistant to *S. aureus* and

MRSA bacteria. In addition, *A. terreus* from various sources can produre metabolites such as acetylaranotin and related natural products; aspernolid D, and furandion, (+) - Geodin asperteron, along with four butenolid and aspernolid B [11]. *A. terreus* also produces enzymes such as invertase and glucosidase when grown in submerged fermentation containing barley black as carbon source; β-xylanase production; cis-aconitic decarboxylase acid (CAD) - an important enzyme in the production of itaconic acid; production of beta-glucosidase, etc. [4]

2. Materials and Methods

2.1. Materials

A. terreus - RTN3 strain has extracted from *A. chinensis* Rosc.-Zingiberaceae family.
Microorganisms used for microbiological testing were obtained from ATCC: *Staphylococcus aureus* ATCC 29213, *MRSA* ATCC 43300.

2.2. Methods

2.2.1. Investigation of antibacterial activity of extracts from fungal and biomass medium Investigation of antibacterial activity of extracts obtained from culture medium of *A. terreus* R-TN3 strain and from lyophilized biomass by diffusion method through paper plates. Taking 10µl of diluent impregnated on a paper plate, leaving in a sterile petri *dish* to evaporate the solvent. Spread the test bacteria on a cotton swab to 20 ml of TSA medium agar. Place the dried solvent paper plate on a freshly spread petri dish. Incubate at 37°C for 24 hours.

2.2.2. *Split the segments for antibacterial activity from crude extract* Thin-layer chromatography (TLC): Proceeding to explore the optimal extraction solvent system for crude extracts from A.terreus culture medium. Raw extracts of A.terreus culture medium were dissolved in MeOH at a concentration of 8mg/ml. Develop with solvent system: $CHCl_3$ - CH_3COOH (9:1). Record results detected by UV254, UV365 and Vanillin sulfuric reagent. Evaluate the separability of the solvent system for the test substance based on the number and characteristics of traces on the chromatogram.

Vacuum column chromatography (VLC): Separates a mixture into different polarized segments so that more pure fractions can be obtained containing antimicrobial active ingredients. The stages of vacuum column chromatography include: mobile phase survey, column preparation and deployment, evaporation of solvents resulting in dry bites or concentrated solutions.

2.2.3. Determination of antioxidant ability by DPPH of MM2 compound Determination of antioxidant ability by DPPH test of MM_2 compound. The results showed that MM_2 has a relatively high antioxidant activity, the ability of catching DPPH free radicals increases linearly with concentration.

2.2.4. Methods for determining the chemical structure of a compound The substances obtained through column chromatography and thin-layer chromatography are processed to determine the structure of the compounds based on spectral methods: Electronic atomizing mass spectrometry (ESI-MS); One-way magnetic resonance spectrum (1H-NMR, 13C-NMR, DEPT) and bidirectional (HMBC, HSQC, COZY, NOESY).

Identification: If a foreign substance appears in the sample, the mass spectrometry can identify its unique chemical structure. The structure of this substance is then compared to a structural library of known substances. If the corresponding substance can not be found in the library, we can obtain a new data and contribute to the structural library after taking further measures to identify the new compound correctly.

2.2.5. Survey of inhibition of cancer cell lines ability of MM2 compound Determination of MCF-7 inhibition of breast cancer cell lines, Hela cervical cancer, Hep G2 and NCI-H460 lung cancer of MM2 compound.
2.2.6. Statistical analysis of data Data is processed statistically by Microsoft Excel software, in Microsoft Office version 2003.

3. Results and discussions

3.1. Investigation of antibacterial activity of extracts from fungal and biomass medium
Investigation of antibacterial activity of extracts obtained from culture medium of *A. terreus* R-TN3 strain and from lyophilized biomass by diffusion method through paper plates. (Table 1).

Table 1. Antibacterial effect of extracts of strain *A. terreus* R-TN3.

Reagent	Suppressive ring diameter (mm)	
	S. aureus	**MRSA**
Extract from culture solution	17.33 ± 0.93 [a]	17.33 ± 0.93 [a]
Extracted from biomass	0.0[b]	0.0[b]

[a] Notes are referenced using alpha superscripts.
[b] Self-supporting.

Extracts from culture solution *A. terreus* R-TN3 medium have antibacterial effects on *S. aureus* and MRSA highly. Extracts from biomass of *A. terreus* R-TN3 strain did not have an antibacterial effect on *S. aureus* and MRSA. This indicates that the antibacterial active ingredients from *A. terreus* R-TN3 strain are extracellularly produced.

3.2. Refine the sample by Vacuum column chromatography (VLC)
Perform high-chromatographic column chromatography with chloroform-methanol solvent system with the rate of change in the direction of increasing methanol, dot and combine the segments and proceed to crystallize the compound. The total high mass of all nine fractions obtained through column chromatography was 10.6g. From segment 1, it is purified to be light yellow, amorphous MM_2 compound, with Rf of 0.692.

3.3. Determination of antioxidant ability by DPPH of MM2 compound
Determination of antioxidant ability by DPPH test of MM_2 compound. The results showed that MM_2 has a relatively high antioxidant activity, the ability of catching DPPH free radicals increases linearly with concentration. (figure. 1).

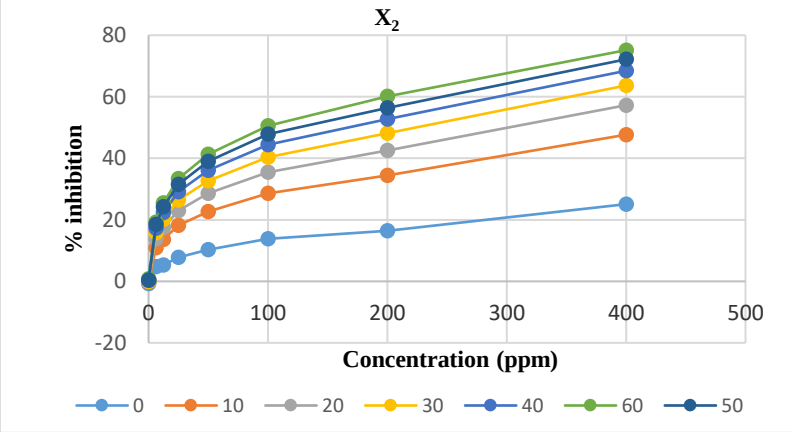

Figure 1. Diagram showing the antioxidant capacity by DPPH test of MM_2 substance.

The results showed that MM_2 has highly antioxidant activity, the ability of catching DPPH free radicals increases linearly with concentration. At a concentration of 400 ppm, MM_2 has the ability to catch more than 75% of DPPH free radicals.

3.4. Methods for determining the chemical structure of MM2 compound

MM_2 compounds obtained from column chromatography, structure is determined by spectroscopic techniques such as H, C_{13}, MS spectroscopy and bidirectional spectroscopy to determine the structure. The isolated MM_2 compound is in the form of powder, light yellow. Soluble in methanol and DMSO, insoluble in n-hexane.

MS spectrum in the form of negative for the tip of the fake molecule ion with *m/z:* $[M-H]^-$ = 398.8, allows determining the mass of MM_2 is 400 corresponding to the molecular formula is $C_{15}H_{14}BrNO_5S$. 13C-NMR spectrum (125 MHz, DMSO-d_6, δ ppm) combined with DEPT90, DEPT135 shows that MM_2 has 9 carbon: 1 carbon carbonyl (ester) at δ_C 165.6, 3 quadruple carbon aromatic oxygen adjacent rings, 1 quadratic aromatic carbon, 2 aromatic aromatic methons corresponding to 2 aromatic protons met meta at δ_H 6.93 (1H, d, J = 2.0 Hz, H-3) and 6.72 (1H, d, J = 1.5 Hz, H-5) and 2 carbon oxymethyl corresponding to 6 protons at δ_H 3.66 (3H, s, H-8) and 3.67 (3H, s, H-9); shows that MM_2 is a derivative of 2,4,6-trihydroxybenzoic acid [12].

In addition, MM_2 also has 1 carbonyl (ketones) at δ_C 200.2, 1 carbon quadrant aromatic oxygen adjacent to δ_C 158.6, 2 carbon quaternary aromatic carbon, 1 carbon methyl corresponding to 3 protons at δH 2.44 (3H, s, H-6 ′) and quaternary aromatic carbon by nitrogen at δC 141.7 together with 2 protons of the second order amine group at δ_H 11.73 (1H, s, NH2); proves that MM2 has cyclopentadienon ring [5], carrying 4 substituents, including 1 methyl group, 1 amino group II, 1 hydroxy group. What about one substituent, from the calculation of the molecular formula from the MS spectrum, shows that MM_2 contains Br and S, so the other substituent is brom and this ring is cyclopentadienthion.

HMBC spectroscopy showed that 2 protons at δ_H 6.93 (H-3) and 6.72 (H-5) interacted with the carbon quadrant aromatic oxygen adjacent to δ_C 158.6 and 1 carbon quadrant aromatic at δ_C 125.4, so these 2 carbon must be C-4 and C-1 on benzoic acid frame. In addition, the proton methine at δ_H 6.72 (H-5) and the three protons of the oxymethyl group at δ_H 3.66 (3H, s, H-8) interact with the carbon quadrant aromatic oxygen adjacent to δ_C 156.8, proves that the C-6 carbon has been oxidized. Similarly, the proton methine at δ_H 6.93 (H-3) and 3 protons of the oxymethyl group at δ_H 3.67 (3H, s, H-9) interact with carbon carbonyl at δ_C 165.6, demonstrating the carbonyl C-7 has been methylated. So MM_2 has a frame that is methyl 2,4-dihydroxy-6-methoxybenzoate. (Table 2)

Table 2. Spectral results of MM_2.

Carbon number	CH_n	Chem. Shifts	Conf. Limits
1	C	200.23	39.2
2	C	160.81	4.8
3	C	111.43	5.2
4	C	132.26	15
5	C	147.44	6.2
8	C	161.87	4.8
9	C	111.88	5.3
10	C	166.16	4.8
11	CH	101.33	2.7
12	C	160.21	4.2
13	CH	103.66	3.2
15	CH_3	12.6	5.1
17	C	171.23	3.5
20	CH_3	56.17	0.9
23	CH_3	52.55	0.3

On the other hand, 3 methyl protons at δ_H 2.44 (3H, s, H-6 ′) interact with 1 aromatic quadratic carbon at δ_C 112.2 and aromatic quadratic carbon adjacent to nitrogen at δ_C 141.7, These 2 carbon should be C-3 ′, C-5 ′. Therefore, the bromine group attaches to C-3′ and the secondary amino group attaches to C-5′. Inferred, this methyl group attaches to the remaining aromatic quadratic carbon at δ_C 128.0 (C-4′) and the aromatic quadratic aromatic carbon adjacent to oxy δ_C 158.6 is C-2 ′ and attached to the benzoic acid framework. via C-2′. (Table 3).

Table 3. Spectral interpretation of MM_2.

J	Group 1	Group 2	Value	Error	Group	nH	Shift	Error
$_4$**J**	11	13	2.20	---	11	1	5.84	0.21
$_5$**J**	11	20	0.30	---	13	1	6.10	0.19
$_2$**J**	15	15	2.88	8.40	15	3	2.46	0.44
$_2$**J**	20	20	9.40	2.80	16.21	3	7.31	2.51
$_2$**J**	23	23	9.40	2.80	20	3	3.88	0.14
					23	3	3.82	0.03

From the HRMS, ^{1}H, ^{13}C-NMR, spectral data, combined with HMBC spectra and compared with references [2], [10], [13]; we identified MM_2 as Methyl 2-((4-amino-2-bromo-3-methyl-5-thioxocyclopenta-1,3-dien-1-yl)oxy)-4-hydroxy-6-methoxybenzoat. This is a new and unpublished substance in any project in the world. (figure. 2).

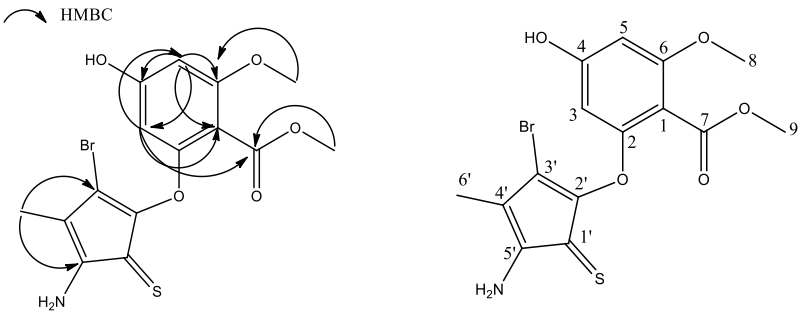

Figure 2. Primary HMBC interactions and structural formulas of MM_2.

3.5. Survey of inhibition of cancer cell lines ability of MM2 compound

Determination of MCF-7 inhibition of breast cancer cell lines, Hela cervical cancer, Hep G2 and NCI-H460 lung cancer of MM_2 compound. Results are presented in Table 4.

Table 4. Results of inhibiting cancer cell lines of compound MM_2.

Concentration	Percentage of cell suppression (%)			
(µg/ml)	MCF-7	Hela	Hep G2	NCI-H460
100	78.21		86.82	
75	74.82	65.81	75.39	71.42
50	66.89	58.20	56.77	46.14
25	24.97	21.07	9.29	19.98
10	-8.45	-4.19	0.75	-1.50
IC_{50} (µg/ml)	**42.26**	**39.21**	**48.30**	**50.26**

The results showed that MM_2 compound's ability to inhibit the test cancer cell lines increased linearly with concentration. At a concentration of 100µg/ml, MM_2 compound inhibits nearly 80% of cancer cell lines and is highest in HepG2 cancer cell. The results showed that the compound MM_2 was able to inhibit the proliferation of in vitro cancer cell lines.

After conducting research, we cultured and extracted a substance containing a crude extract that acts against *S. aureus* and *MRSA* of *A. terreus* R-TN3 strain. The results showed that the compound MM2 was able to inhibit many different cancer cell lines, through the ability to inhibit 4 experimental cell lines. In normal cell lines (Soma), this compound has a higher IC50 value and is outside the cell suppression threshold (> 4 mg/ml). This proves that this compound is highly safe, does not inhibit normal cells. This new compound has not been announced on any works in the country and around the world. This study is completely in line with previous studies on the ability to inhibit cancer cells of *A. terreus* fungi. However, the source of *A. terreus* isolates of previous studies is from soil, water, mangroves, sea, etc. 2014, Suja et al. also demonstrated that *A. terreus* mushroom extract isolated from the ocean was resistant to cancer.

5. Conclusions

The study isolated the pure MM2 compound and spectra to find the structure of MM2 compound with the name: Methyl 2-((4-amino-2-bromo-3-methyl-5-thioxocyclopenta-1,3-dien-1-yl)oxy)-4-hydroxy-6-methoxybenzoat. This is a new compound that has not been published in any works in the world. MM2 compound has antioxidant activity, the ability of catching DPPH free radicals increases linearly with the concentration, at a concentration of 400ppm MM2 has the ability to catch more than 75% of DPPH free radicals. In addition, the compound MM2 also has the ability to inhibit 4 test cell lines: MCF-7 breast cancer, Hela cervical cancer, Hep G2 liver cancer and NCI-H460 lung cancer. The results showed that MM2 compound's ability to inhibit the cancer cell lines increased linearly, at the concentration of 100 µg/ml of MM2 compound, which inhibited nearly 80% of the cancer cell lines and was high. Most in liver cancer cell line HepG2 (at a concentration of 100µg/ml inhibits 86.82% of HepG2 liver cancer cells). This proves that this compound is highly safe, does not inhibit normal cells and has high potential for application.

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References

[1] Dewi R T and Tachibana S 2012 *J. Microb. Biochem. Technol* **04**.
[2] Gaspar A, Cagide F and Quezada E 2013 *Magnetic resonance in Chemistry* **51** 251–254.
[3] Gu W and Qiao C 2012 *Chem. Pharm. Bull.* **60** 1474–1477.
[4] Jain P and Pundir R K 2010 *J. Pharm. Sci. Rev. Res*. **5(3)** 72-76.
[5] Liu J L and Li X H 2015 *Phytochemistry* **12** 27–30.
[6] Macedo F C, Andre L M and Anita JM 2004 *Tetrahedron Letters* **45** 53–55.
[7] Monks A 1991 *Journal of National Cancer Institute* **11 (83)** 757-766.
[8] Pawlak M and Marcin B 2012 *Chemical And Process Engineering* **33(4)** 651-665.
[9] Rao KV and Sadhukhan AK 2000 *Tokyo* **48(4)** 559-562.
[10] Reis J, Gaspar A and Borges F 2014 *Journal of Molecular Structure*, **1056** 31–37.
[11] Shurygina M P and Kurskii Y A 2008 *Tetrahedron* **64** 9784–9788.
[12] Sun Z H and Zhang C F 2010 *Chinese Journal of Natural Medicines* **8(4)** 0244-0246.
[13] Youssef T A, Ramadan M A and Khalifa A A 1998 *Phytochemistry* **49(8)** 2579-2583.