CHEMICAL COMPOSITION AND ANTIFUNGAL ACTIVITY OF *Annona reticulata* L.

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Abstract

Annona reticulata L. is a small deciduous tree species of Annonaceae, traditionally used for treatment of various ailments. The present study investigated the chemicals of the leaves of Annona reticulate and identified three compounds, including taraxerol (1), friedelin (2), and myricetin-3-O-α-L-rhamnopyranoside (3), using NMR spectroscopy. Among them, friedelin (2) is isolated first time from this species. The extracts and purified compound (3) were subjected to the examination of their effects on fungal activity with two fungi strains (Fusarium oxysporum and Pyricularia oryzae) using disc diffusion method. The results demonstrate beneficial effects of Annona reticulata as the antifungal activity for medicinal usages.

Keywords: *Annona reticulata L., antifungal activity, chemical composition.*

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NGHIÊN CỨU THÀNH PHẦN HÓA HỌC VÀ KHẢ NĂNG ỨC CHẾ SINH TRƯỞNG NẤM CỦA LÁ CÂY BÌNH BÁT (*Annona reticulata* L.)

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Tóm tắt

Cây bình bát (Annona reticulata L.) là một loài cây thân gỗ rụng lá thuộc họ Annonaceae, thường được sử dụng để điều trị các bệnh khác nhau. Trong nghiên cứu này, kết quả khảo sát thành phần hóa học của lá cây bình bát, ba hợp chất: taraxerol (1), friedelin (2) và myricetin -3-O-α-L-rhamnopyranoside (3), đã được phân lập và nhận danh bằng phổ NMR. Trong đó, hợp chất friedelin (2) lần đầu tiên được phân lập từ loài cây này. Các cao tổng, cao phân đoạn cùng với hợp chất phân lập được (3) đã được kiểm tra khả năng ức chế sinh trưởng nấm trên hai chủng nấm Fusarium oxysporum và Pyricularia oryzae bằng phương pháp khuếch tán đĩa thạch. Kết quả đã chứng minh tác dụng hiệu quả trong việc ức chế sinh trưởng nấm của lá cây bình bát.

Từ khóa: *Cây bình bát, kháng nấm, thành phần hóa học.*

1. Introduction

Plants have a noteworthy position in the medicinal field due to their therapeutic properties and prove to be a rich source of drugs. The *Annona* genus (Annonaceae) consists of about 119 species, most of which are shrubs and trees widely distributed in the tropical and subtropical regions, including the Southeast Asia countries such as Malaysia, Indonesia, Thailand, Cambodia, Laos, and Vietnam. *Annona reticulata* L. is a small deciduous tree species of Annonaceae, traditionally used for treatment of various ailments (Craker, 2007). Plants contain abundant amount of secondary metabolites and are considered a principal source of therapeutically active compounds along with medicinal formulations. Dopamine, salsolinol, coclaurine, sesquiterpenes and acetogenin are the bioactive metabolites commonly present in leaves (Ogunwande et al., 2006). The leaves showed antipyretic, antihyperglycemic and antiulcer properties (Jamkhande & Wattamwar, 2015). The present study is to explore the phytochemical constituents and antifungal property of *Annona reticulata* (L).

2. Experimental

2.1. Chemicals and reagents

NMR spectra were carried out on a Bruker AM500 FTNMR spectrometer (Bruker, Karlsruhe, Germany) using deuterated solvents at Institute of Chemistry - Vietnam Academy of Science and Technology, Hanoi, Vietnam. IR spectra were recorded on FT/IR- 4600 (Jasco, Japan) at Department of Chemistry, College of Natural Sciences, Can Tho University. TLC was performed on silica gel 60 F_{254} (0.063–0.200mm, Merck, Germany). The zones were detected using UV at 254 or 365 nm or a solution of $FeCl_3$/EtOH or H_2SO_4/EtOH. Column chromatography was performed on silica gel (240-430 mesh, Merck, Germany).

Solvents utilized including *n*-hexane, chloroform, ethyl acetate, methanol (purity ≥ 99.0%), and ethanol 96% were purchased from Chemsol company (Vietnam).

Clean bench (Class II BSC, Esco, Indonesia), autoclave (HVE-50, Hirayama, Japan), centrifuge (Mikro 12-24, Hettich, Germany), was measured for antifungal activity.

2.2. Sample treatment and preparation

The leaves of *Annona reticulata* L. were collected on November 2022 from Soc Trang city and authenticated by Dr. Nguyen Thi Kim Hue. A voucher specimen is kept at the Department of Biology, College of Natural Sciences, Can Tho University, under the number: AnR191122.

The sample was then washed away from muds and dust; the rotten and damaged parts were also discarded. The raw materials were left to dry in the shade at room temperature for some days and then dried in an oven at about 50C until well-dried.

2.3. Extraction and isolation

The fresh leaves of *A. reticulata* (26 Kg) were cleaned thoroughly with water and shade dried in clean and dust-free environment. Dried leaves were finely powdered by using dry grinder. The dried powdered leaves of *A. reticulata* (5.0 Kg) were extracted with ethanol at room temperature and concentrated under reduced pressure to yield a black crude ethanol extract (380 g). This crude extract was suspended in water (1:1, v/v) and successively partitioned with *n*-hexane, ethyl acetate (EtOAc) and water. The resulting fractions were concentrated under reduced pressure to give the corresponding solvent-soluble fractions *n*-hexane (170 g), EtOAc (86 g), and water solubles (36 g).

The *n*-hexane fraction (170 g) was chromatographed on a silica gel column, using solvent gradients of *n*-hexane-EtOAc (1:0, 40:1, 20:1, 10:1, and 5:1, v/v) to afford 5 subfractions (H1-H5). The subfraction H4 was rechromatographed on silica gel column, eluting with $CHCl_3$-EtOAc (20:1, v/v) to yield compound **1** (15.0 mg) and sub-fraction H4.3, which was further purified on a silica gel column eluting with $CHCl_3$-EtOAc (10/1, v/v) to get compound **2** (7.6 mg).

The EtOAc fraction (86 g) was chromatographed on a flash silica gel column (400 – 630 mesh) eluting with gradients of EtOAc-MeOH (from 9:1 to 0:100, v/v) to afford 7 subfractions (E1-E7). The subfraction E3 was subjected to column chromatography on silica gel eluting with EtOAc-MeOH (from 7:3 to 0:100, v/v) to afford 9 subfractions E3.1-E3.9). The subfraction E3.2 was rechromatographed over a RP-18 column eluting with MeOH-H_2O (1:2) to yield compound **3** (23.0 mg).

2.4. Antifungal activity

Antifungal activities of the plant extracts and isolated compound were tested using agar well diffusion method. The prepared culture plates were inoculated with the isolated fungal. Wells were made on the agar surface with 6 mm cork borer. The extracts and isolated compound were poured into the well using sterile syringe. The plates were incubated at 28°C for 7 days. At the end of this period, all the plates were observed for any zone of inhibition. The result was read by observing the zone of inhibition of fungal growth in each plate. The diameter of the inhibition was measured and recorded respectively. The percentage of inhibition of mycelial growth was calculated according to the following formula: (%) = dc − dt/dc×100; where dc (mm) was the mean colony diameter for the control sets and dt (mm) was the mean colony diameter for the treatment sets. The Minimum Inhibitory Concentration (MIC) was determined as the least concentration that showed an inhibitory effect on test organism (Ye et al., 2013; Salama & Marraiki, 2010).

The tested fungi in this study consisted of *Fusarium oxysporum* (a large species complex of both plant and human pathogens), and *Pyricularia oryzae* (causes important diseases in many rice growing regions).

2.5. Statistical analysis

The variation in a set of data has been estimated by performing one-way analysis of variance (ANOVA). Results were calculated from three independent experiments and are shown as mean ± SD, n=3. Results were considered as statistically significant when p value was < 0.05.

3. Results and discussion

3.1. Structure elucidation

The structures of isolated compounds were characterized NMR spectra and by comparison with literature data.

3.1.1. Compound 1

Compound **1** was characterized as white powder, m.p. 282-284°C; = + 9.2 (c 0.1, $CHCl_3$).

TLC results showed the R_f values of 0.17, 0.44 and 0.74 using the respective eluents of PE: DC (60:40, v/v), PE: DC (20:80, v/v) and DC (100%).

^{1}H-NMR (500 MHz, $CDCl_3$), δ_H (ppm): 5.53 (1H, *dd*, *J* = 8.5 and 3.5 Hz, H-15); 3.19 (1H, *dd*, *J* = 10.5 and 3.5 Hz, H-3); 1.09 (3H, *s*, H-27); 0.97 (3H, *s*, H-23); 0.95 (3H, *s*, H-29); 0.92 (3H, *s*, H-25); 0.91 (3H, *s*, H-28); 0.91 (3H, *s*, H-30); 0.82 (3H, *s*, H-26); 0.80 (3H, *s*, H-24).

^{13}C-NMR (125 MHz, $CDCl_3$), δ_C (ppm): 158.1 (C-14); 116.9 (C-15); 79.1 (C-3); 55.5 (C-5); 49.3 (C-9); 48.7 (C-18); 41.3 (C-7); 39.0 (C-8); 38.7 (C-4); 38.0 (C-17); 37.7 (C-1); 37.7 (C-12); 37.5 (C-10); 36.7 (C-16); 35.8 (C-13); 35.1 (C-19); 33.7 (C-21); 33.3 (C-29); 33.1 (C-22); 29.9 (C-28); 29.8 (C-26); 28.8 (C-20); 28.0 (C-23); 27.1 (C-2); 25.9 (C-27); 21.3 (C-30); 18.8 (C-6); 17.5 (C-11); 15.4 (C-25); 15.4 (C-24).

Compound **1** was obtained from *n*-hexane extract as white powder, m.p. 282-284°C (MeOH). IR spectrum (KBr, ν_{max}, cm^{-1}): 3435 (-OH), 2941, 2869, 1642, 1457, 1381, 1030. The ^{13}C-NMR and DEPT spectra of **1** displayed 30 carbon signals, including 1 carbinol carbon >CH-OH at δ_C 79.1 (C-3), one tertiary olefinic carbon = CH- [δ_C 116.9 (C-15)], one quaternary olefinic carbon =C< [δ_C 158.1 (C-14)], 8 methyl carbons -CH_3, 10 methylene carbons, 3 methine carbons >CH- [δ_C 55.5 (C-5); 49.3 (C-9) and 48.7 (C-18)], and 6 quaternary carbons. The ^{1}H-NMR signals suggested the presence of triterpene skeleton – taraxerane, consisting of one hydroxyl group and one double bond. From HMBC, 2 methyl groups at C-29 and C-30 correlated with each other and also correlated with C-20, C-19 and C-21. Both methyl groups H_3-26 and H_3-27 correlated with the olefinic quaternary carbon at C-14. In addition, protons H_3-26 also correlated with C-8, C-9 and C-7. Protons H_3-27 also correlated with carbons at C-13, C-18 and C-12. Besides, olefinic proton at δ_H 5.53 corresponded with three quaternary carbons at C-8, C-13 and C-17; so the double bond must be at C-14 and C-15. Proton H_3-28 corresponded with four carbons at C-17, C-18, C-16 and C-22. Methyl proton H_3-25 correlated with three carbons at C-9, C-5 and C-1. Two methyl groups C-23 and C-24 correlated with each other and also correlated with carbon at C-4, C-5 and C-3 (**Figure 1**). The double of doublet signal (*J* = 8.5 and 3.5 Hz) appeared at δ_H

5.53 is assigned to the olefinic proton H-15. The broad doublet signal (J = 10.5 and 3.5 Hz) was found at δ_H 3.19 could be assigned to proton H-3. This allow to identify compound **1** as β-taraxerol through the comparison of physical and spectral data with the published data (Koay et al., 2013).

3.1.2. Compound 2

Compound **2** was characterized as white crystals, m.p. 260-262°C.

TLC results showed the R_f values of 0.22, 0.35 and 0.90 using the respective eluents of Hex: C (80:20, v/v), Hex: Ac (95:5, v/v) and Hex: EtOAc (90:10, v/v).

^{1}H-NMR (500 MHz, $CDCl_3$) **δ_H** (ppm): 1.18 (3H, *s,* H-28); 1.05 (3H, *s*, H-27); 1.01 (3H, *s*, H-26); 1.00 (3H, *s*, H-29); 0.95 (3H, *s*, H-30); 0.89 (3H, *d*, J = 6.5 Hz; H-23); 0.87 (3H, *s*, H-25); 0.73 (3H, *s*, H-24).

^{13}C-NMR (125 MHz, $CDCl_3$) **δ_C** (ppm): 213.2 (C-3); 59.5 (C-10); 58.3 (C-4); 53.3 (C-8); 42.8 (C-18); 42.2 (C-5); 41.5 (C-2); 41.3 (C-6); 39.7 (C-13); 38.3 (C-14); 37.5 (C-9); 36.0 (C-16); 35.7 (C-11); 35.4 (C-19); 32.8 (C-21); 32.5 (C-15); 32.1 (C-22); 32.1 (C-28); 31.8 (C-29); 30.5 (C-12); 35.0 (C-30); 30,0 (C-17); 28,2 (C-20); 22.3 (C-1); 20.3 (C-26); 18.7 (C-27); 18.3 (C-7); 18.0 (C-25); 14.7 (C-24); 6.8 (C-23).

The molecular formula of compound (**2**) was established as $C_{30}H_{50}O$ by the pseudo molecular $[M+H]^+$ ion peak at *m/z* 427.2 in ESI-MS analysis. The ^{13}C-NMR spectra showed characteristic resonances for 30 carbons, including 8 methyl carbons -CH_3, 11 methylene carbons, 4 methine carbons >CH-, and 7 quaternary carbons. In addition, the ^{1}H-NMR showed seven methyl groups appearing as a singlet and one methyl doublet, all in the up-field region ranges between δ 0.73 and 1.18 ppm. The ^{13}C-NMR data of the group of high-intensity signals present a signal at δ_C 213.2 which is characteristic of a carbonyl carbon. The methine protons appeared at C-4 at δ 2.23 ppm, at C-8 as a multiplet at δ 1.39 ppm, and at C-18 as a multiplet at δ 1.57 ppm. The HMBC spectra also showed a correlation of methyl protons at δ_H 0.89 (H-23, *d*, J = 6.5 Hz) with C-5 (δ 42.2 ppm) and C-3 (δ 213.2 ppm), a correlation of other methyl protons (H-28, δ 1.18 ppm) with C-16 (δ 36.0 ppm), C-17 (δ 30.0 ppm) and C-22 (δ 32.1 ppm), while methyl protons (H-26, 27, δ 1.01, 1.05 ppm) showed correlations with C-13 (δ 39.7 ppm), C-14 (δ 38.3 ppm) and C-15 (δ 32.5 ppm) (**Figure 1**). The methylene protons (H-2, δ 2.32/2.38 ppm) showed correlations with carbonyl carbon (C-3, C=O, δ 213.2 ppm), and methine carbon (C-10, δ 59.5 ppm). The structure of friedelin (**2**) was identified by comparing the spectra data with previously reported data (Sousa et al., 2012)

3.1.3. Compound 3

Compound **3** was obtained as a yellow needles, m.p. 197-199°C.

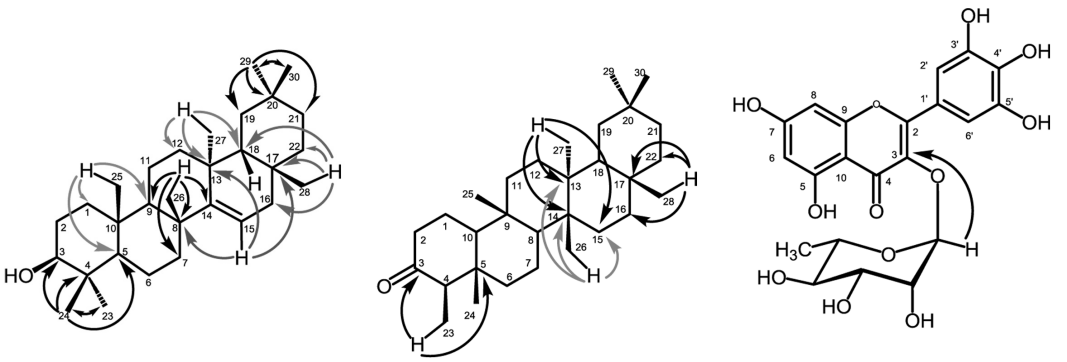
TLC results showed the R_f values of 0.22, 0.50 and 0.75 using the respective eluents of Hex: EtOAc (40:60, v/v), C: MeOH (85:15, v/v) and C: Ac (30:70, v/v).

^{1}H-NMR (500 MHz, CD_3OD**)**, δ_H (ppm): 6.99 (2H, *s*, H-2′, 6′); 6.40 (1H, *d*, J = 2.0 Hz, H-8); 6.24 (1H, *d*, J = 2.0 Hz, H-6); 5.36 (1H, *s*, H-1′′); 4.23 (1H, *s*, H-2′′); 3.81 (1H, *dd*, J = 9.3 and 3.3 Hz, H-3′′); 3.52 (1H, *m*, H-5′′); 3.33 (1H, *m*, H-4′′); 1.00 (3H, *d*, J = 6.0 Hz, H-6′′).

^{13}C-NMR (125 MHz, CD_3OD), δ_C (ppm): 179.6 (C-4); 165.8 (C-7); 163.2 (C-5); 159.3 (C-2); 158.4 (C-9); 146.9 (C3′, 5′); 137.9 (C-4′); 136.2 (C-3); 121.9 (C-1′); 109.6 (C2′, 6′); 105.6 (C-10); 103.5 (C-1′′); 99.8 (C-6); 94.7 (C-8); 73.3 (C-4′′); 72.1 (C-3′′); 72.0 (C-5′′); 71.8 (C-2′′); 17.8 (C-6′′).

Compound **3** was obtained as yellow needles. The ^{1}H-NMR spectrum exhibited a characteristic *meta*-coupled proton signal at δ 6.24 (1H, *d*, J = 2.0 Hz) and δ 6.40 (1H, *d*, J = 2.0 Hz) corresponding to H-6 and H-8 of flavonoid A ring. The other AX coupling system at δ 6.99 (2H, *s*) was assigned to H-2′ and H-6′ of B ring. In addition, there is the appearance of an α-L-rhamnopyranosyl moiety. Therefore, the ^{1}H-NMR spectrum of **3** showed the presence of an anomeric proton signal at δ 5.36 (1H, *s*), a methyl signal at δ 1.00 (3H, *d*, J = 6.0 Hz) and of six additional carbon signals at δ 103.5 (C-1′′), 73.3 (C-4′′), 72.1 (C3′′), 72.0 (C-5′′), 71.8 (C-2′′), and 17.8 (C-6′′). The position of the sugar unit in compound **3** was confirmed by the HMBC correlation between H-1′′ (δ 5.36) and C-3 (δ 136.2) (**Figure 1**). By comparing the NMR spectral data with those reported in literature (Nguyen et al., 2015), the structure of **3** was determined as myricetin-3-O-*α*-L-rhamnopyranoside or myricitrin.

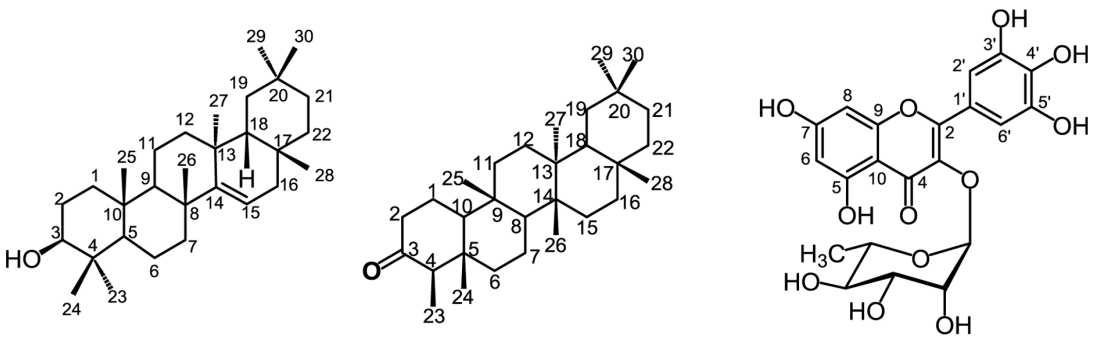
Three compounds **1-3** were isolated and identified from the leaves of *Annona reticulata* L., including taraxerol (**1**), friedelin (**2**), and myricetin-3-O-*α*-L-rhamnopyranoside (**3**) by analysis of their NMR spectra and comparison with literature data (**Figure 2**). The compound (**2**) is isolated first time from this species.



1 **2** **3**

Figure 1. Some typical HMBC correlations of compound (1) – (3).

Note: ↷ *show typical H-C heteronuclear multiple bond correlation*



Taraxerol (1) **Friedelin (2)** **Myricitrin (3)**

Figure 2. Structures of compounds (1) – (3)

3.2. *In vitro* antifungal results

Table 1. The percen tage of inhibition and MIC values of extracts and isolated compound

Extracts and isolated compounds		The percentage of inhibition at different concentrations (%)					
Extracts (µg/mL)		**100**	**500**	**1000**	**1500**	**2.000**	**3000**
Crude extract	*F. oxysporum*	$2.3^{f}\pm1.4$	$5.8^{e}\pm1.9$	$9.8^{d}\pm2.0$	$47.8^{c}\pm2.5$	63.7 ± 1.3	$100.0^{*a}\pm0.0$
	P. oryzae	$3.5^{f}\pm1.9$	$6.4^{e}\pm1.7$	$11.3^{d}\pm3.1$	$50.3^{c}\pm2.2$	$76.8^{b}\pm2.1$	$100.0^{a}\pm0.0$
***n*-Hexane extract**	*F. oxysporum*	$1.8^{f}\pm0.9$	$3.9^{e}\pm1.4$	$7.8^{d}\pm2.6$	$35.6^{c}\pm1.1$	$58.6^{b}\pm1.5$	$68.8^{a}\pm1.2$
	P. oryzae	$14.3^{f}\pm1.2$	$31.4^{e}\pm0.8$	$41.2^{d}\pm2.6$	$50.6^{c}\pm1.2$	$62.6^{b}\pm0.5$	$88.6^{a}\pm0.7$
Ethyl acetate extract	*F. oxysporum*	$42.6^{e}\pm1.7$	$55.8^{d}\pm0.9$	$74.1^{c}\pm0.9$	$87.3^{b}\pm1.8$	$100.0^{*a}\pm0.0$	$100.0^{a}\pm0.0$
	P. oryzae	$27.7^{f}\pm1.6$	$44.6^{e}\pm1.5$	$68.7^{d}\pm2.6$	$77.5^{c}\pm2.1$	$85.2^{b}\pm1.8$	$100.0^{a}\pm0.0$
Isolated compound (µg/mL)		**10**	**20**	**40**	**80**	**160**	**320**
Myricetin-3-O-*α*-L-rhamnopyranoside	*F. oxysporum*	-	$4.5^{e}\pm0.7$	$10.8^{d}\pm2.2$	$21.4^{c}\pm1.4$	$33.8^{b}\pm1.2$	$65.2^{a}\pm0.9$
	P. oryzae	-	-	$8.2^{d}\pm0.8$	$18.6^{c}\pm1.2$	$44.2^{b}\pm1.3$	$58.2^{a}\pm1.3$
Azoxystrobin	*F. oxysporum*	$11.5^{f}\pm1.1$	$23.6^{e}\pm0.9$	$38.2^{d}\pm1.6$	$57.8^{c}\pm1.6$	$74.5^{b}\pm1.3$	$94.4^{a}\pm1.2$

*Note: (-): no inhibitory effect, *: MIC*

different letters (a, b, c, d, e, f) indicate significant differences between treatments ($p < 0.05$)

Antifungal effect of tested extracts and myricitrin compound (**3**) was tested against two selected strains of fungi *Fusarium oxysporum* and *Pyricularia oryzae*. According to results mentioned in **Table 1**, the antifungal activity of the extracts and myricitrin increased linearly with increase in concentration of samples. Our analysis indicated that ethyl acetate extract exhibited potent antifungal effect against *F. oxysporum* with the percentage inhibition of 42.6%, 55.8%, 74.1%, and 87.3% at the concentration of 100, 500, 1000, and 1500 µg/mL respectively and get the MIC value at 2000 µg/ml. Myricitrin compound showed significant inhibition zone against *F. oxysporum* with the percentage inhibition of 65.2% at 320 µg/mL, compared with a standard, positive control (320 µg/mL azoxystrobin) of 94.4%.

A. reticulata is one of the most popular nutritional and medicinal plant which is rich in variety of secondary metabolites including polyphenols. The antifungal effect of ethyl acetate extract against different pathogenic fungal strains had supported traditional claims of plant as a medicine. The presence of phenolic and polyphenolic compounds in the ethyl acetate extract may be attributed to potent inhibitory effect of extract against fungi strains. It is already reported that polyphenolic compounds are biologically active and possesses antimicrobial property suggesting that antifungal activity of ethyl acetate extract may be due to the presence of some phenolic compounds (Bansal et al., 2013). Several mechanisms have been explained for antimicrobial effect of polyphenols that mainly includes ability of polyphenols to neutralize bacterial toxins, biofilm inhibition, reduction of host ligands adhesion, enzyme inhibition, interaction with eukaryotic DNA and membrane disruption (Barile et al., 2007).

The cell wall of the fungi is more complex in lay out than some other microbes like bacteria. In spite of this permeability differences, however, some of the extracts have still exerted degree of inhibition against some fungi as well. In the present study, *A. reticulata* leaves extract possesses antifungal activity against some of the tested fungi and the plant contains potential antifungal component for the therapy of infections. It is concluded that traditional plants may represent new sources of antimicrobials with stable, biologically active components that can establish a scientific base for the use of plants in modern medicine and the compounds are known to be biologically active, therefore, aid the antifungal activity.

4. Conclusion

The results of this study revealed that from the extracts of *Annona reticulata* L. leaves, grown in Soc Trang city, three compounds: taraxerol (**1**), friedelin (**2**), and myricetin-3-O-α-L-rhamnopyranoside (**3**) have been isolated and identified, in which compound (**2)** isolated first time from this species. The structures of these compounds have been elucidated by NMR spectroscopy and in comparison with the literature data. Furthermore, the antifungal potentials of fractioned extracts and isolated compound were also evaluated through the agar well diffusion method. The results indicated that ethyl acetate extract have the good antifungal activity and compound myricitrin (**3**) exhibited the good ability of antifungal activity against *Fusarium oxysporum*.

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