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Antimicrobial activity of the crude extract, fractions and isolation of zerumbone from the rhizomes of *Zingiber roseum*

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ABSTRACT: The rhizome of *Zingiber roseum* has been used as traditional medicines for the treatment of gastric ulcer, skin diseases, and liver infections. Zerumbone is an essential sesquiterpenoid previously isolated from different species of Zingiberaceae and possesses numerous pharmacological properties. Based on previous reports, the aim of the current study was to investigate the antimicrobial activity of extract, fractions, and isolation of zerumbone from *Zingiber roseum*. The antimicrobial activity was determined using disc diffusion method. The serial tube dilution technique was used to determine Minimum Inhibitory Concentration (MIC) of the samples. Vacuum Liquid Chromatography (VLC) and open column chromatography were used for separation. The methanol extract exhibited maximum antimicrobial activity against *Escherichia coli*, *Saccharomyces cerevisiae*, and *Aspergillus niger* in both methods. VLC of the extract yielded five fractions (Fr. 1 to Fr. 5). Fr. 1 exhibited maximum antimicrobial activity compared to the other fractions. Column chromatography of Fr.1 generated 47 fractions. Fraction 1 to 9 were combined and crystallized in n-hexane to isolate zerumbone as the most abundant source (yield 6.5 % from dried MeOH extract). Zerumbone showed good activity against all tested bacteria. The results of our study might validate the ethnobotanical use of *Zingiber roseum* rhizomes in the treatment of infectious diseases. Moreover, *Zingiber roseum* could be one of the most abundant sources for the isolation of natural zerumbone to study different pharmacological studies in the field of new drug discovery.

KEYWORDS: *Zingiber roseum*; antimicrobial activity; MeOH extract; VLC fractions; zerumbone.

1. INTRODUCTION

As per of continuing our research in the field of new drug discovery from medicinal plants, we investigated *Zingiber roseum* (Family: Zingiberaceae) [1, 2]. The rhizomes of *Zingiber roseum* are used by folk practitioners in Gazipur and Tangail district of Bangladesh for the treatment of gastric ulcer, asthma, wound and rheumatic disorders. According to literature, the rhizomes are also used as traditional medicines for the treatment of fever, cough, skin diseases, indigestion and liver infections [3- 5]. There is very limited number of pharmacological activities have been reported about this plant such as antifertility, antispasmodic, antioxidant and myorelaxant activities [5]. Some essential oils of *Zingiber roseum* have been published in one literature [6]. In addition, the ethanol extract of *Zingiber roseum* rhizomes has been investigated for antimicrobial activities on five microorganisms [7].

Zerumbone is a sesquiterpenoid commonly isolated from different species of Zingiberaceae family. It is the major constituent of essential oils of *Zingiber zerumbet* and has been also isolated from *Zingiber aromaticum*

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[8-10]. In our previous study, we isolated zerumbone as an antiulcer principle from *Zingiber montanum* [11]. The anti-ulcer effect was comparatively higher than existing products in the market. Zerumbone has numerous pharmacological properties including antitumor [10], antinociceptive [12], anti-inflammatory [13] and HIV inhibitory activities [14]. It induces apoptosis, cell cycle arrest, and antimigratory effects in SW480 colorectal cancer cells [15]. As zerumbone possesses numerous therapeutic benefits, it is now one of the targeted compounds in the field of new drug discovery [15, 16]. Based on previous reports of *Zingiber roseum* and zerumbone, the present study has been conducted to investigate the antimicrobial activity of methanolic extract, VLC fractions and isolated compound (**1**). In addition, we were also interested to investigate *Zingiber roseum* as one of the new source of natural zerumbone.

2. RESULTS AND DISCUSSION

Disc diffusion is the most useful method to determine the antimicrobial activity and has been used in this study [17]. As summarized in the Table 1, methanol extract showed antimicrobial activity at the doses of 200 µg/disc and 400 µg/disc against all tested microorganisms. The highest inhibition zone diameter was 13 mm on *Escherichia coli*, *Saccharomyces cerevisiae* and *Aspergillus niger* at a dose of 400 µg/disc, and the lowest inhibition was 8 mm on *Shigella dysenteriae* at a dose of 200 µg/disc. The reference antibacterial agent amoxicillin showed maximum 21 mm zone of inhibition on *Staphylococcus aureus* and lowest 16 mm on *Sarcina lutea* at a dose of 20 µg/disc. The reference antifungal agent nystatin showed maximum 17 mm zone of inhibition on *Candida albicans* and minimum 15 mm on *Saccharomyces cerevisiae*. The results indicate that the methanol extract of *Zingiber roseum* rhizome has both antibacterial and antifungal activities. The result of this study strongly suggested to do further study for the investigation of therapeutic agents of *Z. roseum*. Reported literature described antimicrobial and antifungal activities of *Z. roseum* rhizomes of the ethanol extract at high doses against only five microorganisms [7]. The literature reported the highest zone of inhibition of 10 mm on *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans* at a dose of 5000 µg/disc., and the minimum zone of inhibition of 8 mm on *Micrococcus luteus* at a dose of 5000 µg/disc. Additionally, the minimum inhibitory concentration of *Z. roseum* of the ethanol extract was not reported in the literature.

In this study, we conducted the research of methanol extract on six bacteria and three fungi with low doses. The methanol extract showed highest zone of inhibition (13 mm) at a dose of 400 µg/disc. The studies suggest that the better inhibition of methanol extract might be for the more antimicrobial compounds in methanol extract compared to ethanol extract. It could also be for the different geographical origin of the plant. As the extract showed antimicrobial activities in disc diffusion method and there is no report for the minimum inhibitory cocentration of *Z. roseum* extract, we investigated the minimum inhibitory concentration. The results of the MIC determinations (Table 2) showed noticeable values against the entire set of the tested organisms. The obtained MICs varied from 625 µg/mL to 1.25 mg/mL. The highest MIC value 625 µg/mL was observed against all tested organisms except *Shigella dysenteriae* which showed MIC value 1.25 mg/mL. The positive control amoxicillin showed the maximum MIC value 4 µg/mL against *Pseudomonas aeruginosa* and *Sarcina lutea*. The results of disc diffusion and MIC of extract strongly suggested doing fractionation of the crude extract.

The separation of the MeOH extract by VLC yielded five fractions. All fractions (Fr. 1 to Fr. 5) were assayed for the determination of antimicrobial activity using disc diffusion method. The MIC values of these fractions were also investigated. The results showed that Fr. 1 was found to be the most potent fraction. The hexane fraction showed maximum 16 mm zone of inhibition on *Escherichia coli*, and *Candida albicans* at a dose of 200 µg/disc. The minimum zone of inhibition of Fr.1 was 10 mm on *Shigella dysenteriae*. The highest MIC value of Fr.1 was 256 µg/mL against *Escherichia coli* and *Candida albicans*, and the lowest value was 625 µg/mL against other organisms. Fr.2 and Fr.3 also exhibited moderate activity against all tested bacteria and fungi, which were relatively lower than the most potent fraction (Fr.1). In contrary, Fr .4 and Fr. 5 did not showed activity at the dose of 1,250 µg/mL. Thus, the results indicated that Fr.1 is the most potent fraction which might contain the active antimicrobial compound. Based on the result of fractions, we performed open column chromatography of Fr. 1, and a total of 47 sub-fractions were obtained.

Table 1 : Antimicrobial activity of MeOH extract, VLC fractions and zerumbone **(1)** using disc-diffusion method.

Tested samples	Dose µg/disc	Tested Microorganism									
		Gram-negative bacteria				Gram Positive Bacteria			Fungi		
		Ec	Pa	Sd	Sb	Sa	Sl	Ca	Sc	An
MeOH extract	200	10	9	8	11	9	10	11	12	11
	400	13	11	9	12	11	11	12	13	13
VLC Fractions										
Fr.1	200	16	11	10	12	13	13	16	13	13
Fr.2	200	12	10	10	11	11	12	13	11	11
Fr.3	200	8	9	9	10	9	10	10	11	11
Fr.4	200		-	-	-	-	-	-	-	-
Fr.5	200		-	-	-	-	-	-	-	-
Zerumbone **(1)**	50	12	11	8	9	11	10	7	8	7
	100	16	13	10	11	15	12	8	9	9
Reference compounds										
Amoxicillin	20	18	17	20	18	21	16			
Nystatin	10							17	15	16

(-): No inhibition, VLC fractions: - Hexanes fraction (Fr·1), Dichloromethane fraction (Fr· 2), Ethyl acetate fraction (Fr· 3), Acetone fraction (Fr· 4), Methanol fraction (Fr· 5)·
Tested Microorganisms: Ec: *Escherichia coli*, Pa: *Pseudomonas aeruginosa*, Sd: *Shigella dysenteriae*, Sb: *Shigella boydii*, Sa: *Staphylococcus aureus*, Sl: *Sarcina lutea*, Ca: *Candida albicans*, Sc: *Saccharomyces cerevisiae*, An: *Aspergillus niger*

Journal of Research in Pharmacy
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Table 2· Minimum inhibitory concentration (µg/mL) of the methanolic extract, fractions, zerumbone (**1**), and reference antibiotics.

Tested samples	Tested microorganism								
	Gram-negative bacteria			Gram Positive Bacteria			Fungi		
	Ec	Pa	Sd	Sb	Sa	Sl	Ca	Sc	An
MeOH extract	625	625	1.25	625	625	625	625	625	625
VLC Fractions									
Fr. 1	256	625	625	625	625	625	256	625	625
Fr. 2	625	625	625	625	625	625	625	625	625
Fr. 3	>1250	1250	>1250	1250	>1250	1250	625	625	625
Fr. 4	>1250	>1250	>1250	>1250	>1250	>1250	>1250	>1250	>1250
Fr. 5	>1250	>1250	>1250	>1250	>1250	>1250	>1250	>1250	>1250
Zerumbone (**1**)	64	128	128	128	128	128	256	256	256
Reference compounds									
Amoxicillin	8	4	8	8	8	4			
Nystatin							8	8	16

VLC fractions: - Hexanes fraction (Fr· 1), Dichloromethane fraction Fr· 2), Ethyl acetate fraction (Fr· 3), Acetone fraction (Fr· 4), Methanol fraction (Fr· 5)·

Tested microorganisms: Ec: *Escherichia coli*, Pa: *Pseudomonas aeruginosa*, Sd: *Shigella dysenteriae*, Sb: *Shigella boydii*, Sa: *Staphylococcus aureus*, Sl.: *Sarcina lutea*, Ca: *Candida albicans*, Sc: *Saccharomyces cerevisiae*, An: *Aspergillus niger*

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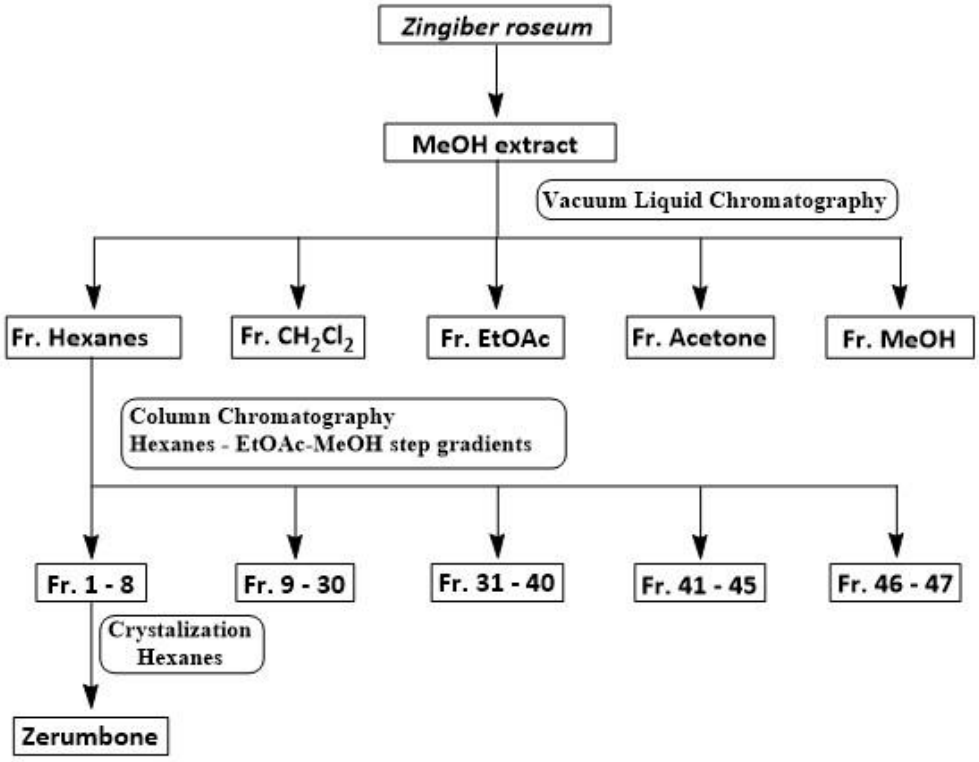

Figure 1. Isolation and purification of zerumbone (**1**) from *Zingiber roseum* rhizome.

Then, based on analytical TLC and visual appearance, sub-fraction 1 to 9 were combined and crystallized with hexanes to yield 9.1 gm colorless crystals of compound **1**. Spectroscopic analysis has been performed to elucidate the structure of isolated compound (**1**). UV spectrum of **1** showed absorption at 213, 250, 302, 319 nm which indicated the presence of a π-π conjugated system in **1**, the presence of carbonyl group was revealed by strong absorption in IR spectrum at 1700 cm^{-1}. In the EI-MS spectrum, the molecular ion peak at m/z 218 suggested a molecular formula of $C_{15}H_{22}O$. In ^1H NMR spectrum, a pair of trans-olefinic protons was observed at δ 5.85 (1H, d, *J* = 16.4 Hz) and 5.96 (1H, d, *J* = 16.4 Hz). The spectrum also showed two olefinic protons at δ 5.23 (1H, br, dd, *J* = 9.2, 7.2 Hz) and 6.00 (1H, br, d, *J* = 11.9 Hz), six methylene protons at δ 1.89 (1H, d, *J* = 12.5 Hz) and 2.20–2.43 (5H, m), and four methyl groups at 1.05 (3H, s), 1.09 (3H, s), 1.52 (3H, s), 1.78 (3H, s). The ^{13}C NMR spectrum showed 15 carbon resonance signals which indicated 1 to be a sesquiterpene deraivative. A characteristice peaks for carbonyl carbon was observed at 204.4 and four cabon signals of methyl groups were appeared at 29.4 (C-15), 24.2 (C-14), 15.2 (C-12) and 11.8 (C-13). Based on these spectral data analyses, **1** was identified as zerumbone (Figure. 2), a cyclic sesquiterpene, which was previously isolated from *Zingiber montanum* in one of our projects as an anti-ulcer principle [11]. ^1H NMR, ^{13}C NMR and EI-MS of **1** is also very consistent with the previously isolated zerumbone [8, 9].

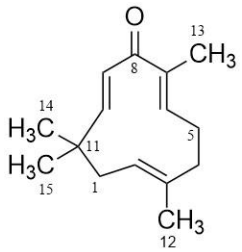

Figure 2. Chemical structure of zerumbone (**1**) isolated from *Z. roseum*.

The zone of inhibition and MIC value of zerumbone were also evaluated. The compound showed good antibacterial activity against all tested gram-positive and gram-negative bacteria. The maximum zone of inhibition of zerumbone was 16 mm on *Escherichia coli* at a dose of 100 µg/disc and the minimum 7 mm on *Candida albicans* as well as *Aspergillus niger* at a dose of 50 µg/disc. The MIC value varied from 64 µg/mL to

256 µg/mL against all tested organisms and the maximum effect (64 µg/mL) was observed against *Escherichia coli*. The results showed that zerumbone exhibited antibacterial activities against all tested gram-negative and gram-positive bacteria. In contrary, the compound showed lower activities against all tested fungi compared to the active fraction.

Finally, the antibacterial activities of the crude extract may be due to the presence of zerumbone. However, the antifungal activity of zerumbone showed that this compound is not the active antifungal principle. Therefore, more compounds needed to be isolated from other sub-fractions to ensure antifungal and antibacterial principles of *Zingiber roseum* rhizomes.

3. CONCLUSION

The results of this study indicated that the methanol extract of *Zingiber roseum* exhibited antimicrobial activity against all tested organisms. VLC fractionation yielded five fractions and Fr.1 showed maximum antimicrobial activity compared to other fractions. The results of extract and fractions strongly recommend the use of rhizomes as traditional medicines for the treatment of infectious diseases. Open column chromatography of the active fraction (Fr.1) yielded zerumbone as the most abundant compound of this plant. Therefore, the yield (6.5 % from dried MeOH extract) strongly suggest the use of *Zingiber roseum* as one of the major sources of natural zerumbone for further study in the field of new drug discovery.

4. MATERIALS AND METHODS

4.1. Drugs and chemicals

Organic solvents were obtained from Merck, Germany. Nystatin and Amoxicillin were obtained from Square Pharma, Bangladesh. Nutrient agar was obtained from Techno Pharmchem, Bahadurgarh, India. Nutrient Broth was obtained from Hi-Media Laboratories Ltd. Mumbai, India. TLC was run on Merck TLC plates precoated with $Si60F_{254}$ with visualization by spraying with 10% H_2SO_4 in MeOH followed by heating. VLC was done using Silica gel 60 (0.040 –0.005 mm), Merck, Germany. Open column chromatography was done by using Silica gel 60 (0.063–0.020 mm), Merck, Germany. The IR Spectrum was obtained using a Shimadzu IR Prestige-2 FT-IR while the ^{1}H-NMR spectra were recorded on an ultra-shield Bruker DPX 400 spectrometer. The NMR spectra were recorded running gradients and using residual solvent peak (δ 7.25 s for ^{1}H NMR, δ 77.23 t for ^{13}C NMR) as an internal reference. EI–MS spectrum was obtained using Agilent GC/MS with MassHunter.

4.2. Plant materials and extraction

The rhizomes of *Zingiber roseum* were collected from Modhupur Garh, Tangail on November 9, 2011, and their identity was confirmed by the National Herbarium of Bangladesh, Chiriakhana Road, Mirpur-1, Dhaka-1216, Bangladesh, where a voucher specimen has been deposited (Accession Number 37515). After collection, the rhizomes were washed with water, cut into pieces and sun-dried. Then, dried rhizomes were powdered by using a Spices Mill. Dried powdered rhizomes (1.3 kg) were extracted with MeOH at room temperature (3.5 L × 72 h × 3 times). The extracting solvent was filtered, and the filtrate was concentrated *in vaccuo* by a rotary evaporator (45 °C) to get the crude MeOH extract (140.0 g, yield: 11.1 % from dried powder).

4.3. Separation of the MeOH extract

The crude MeOH extract (139 g) was subjected to silica gel (275 g) VLC and eluted with hexanes (5 L), dichloromethane (5 L), EtOAc (5 L), acetone (5 L) and MeOH (5 L) to obtain five fractions: Fr. 1 (eluted with hexanes, 27.4 g), Fr. 2 (eluted with DCM, 5.2 g), Fr. 3 (eluted with EtOAc, 19.2 g), Fr. 4 (eluted with acetone, 34.7 g) and Fr. 5 (eluted with MeOH, 18.6 g). Active fraction, Fr. 1 (27.0 g) was subjected to silica gel (80.0 g) open column chromatography and eluted with hexanes-ethyl acetate (Hex-EtOAc) and ethyl acetate-MeOH step gradients, and 47 fractions of 100 mL each were collected as follows: 1–8 (hexanes), 9–30 (Hex-EtOAc, 9.5:0.5), 31–40 (Hex-EtOAc, 9:1), 41–45 (Hex-EtOAc, 8.0:2.0), 46–47 (Hex-EtOAc, 7.5:2.5). Based on the analytical TLC, fraction 1 to 9 were combined and crystallized in *n*-hexane to get colorless crystals of compound **1** (9.1 g, yield: 6.5 % from dried MeOH extract). The schematic diagram of the isolation and purification of zerumbone (**1**) from the MeOH extract are shown in the Fig. 1.

Compound (1): colorless crystals; m.p. 65–67 °C (lit. 67.5–68.0 °C); UV max (MeOH) nm (log ε): 213 (3.77), 250 (3.94), 302 (2.90), 319 (2.91); IR max (KBr) cm^{-1}: 1263.4, 1386.8, 1452.4, 1700.0, 2920.2, 2941.1, 3026.3; ^{1}H NMR ($CDCl_3$, 400 MHz) δ : 1.05 (3H, s, 14-H/15-H), 1.19 (3H, s, 15-H/14-H), 1.52 (3H, s, 12-H), 1.78 (3H, s, 13-H), 1.89 (1H, d, *J* = 12.5 Hz, 1-Ha), 2.20–2.43 (5H, m, 1-Hb, 4-H2, 5-H2), 5.23 (1H, br, dd, *J* = 9.2, 7.2 Hz, 2-H),

5.85 (1H, d, *J* = 16.4 Hz, 10-H), 5.96 (1H, d, *J* = 16.4 Hz, 9-H), 6.00 (1H, br, d, *J* = 11.9 Hz, 6-H); ^{13}C NMR ($CDCl_3$, 500 MHz) δ : 204.4 (C-8), 160.8 (C-10), 148.9 (C-6), 138.0 (C-7), 136.3 (C-3), 127.2 (C-9), 125.0 (C-2), 42.4 (C-1), 39.4 (C-4), 37.9 (C-11), 29.4 (C-15), 24.4 (C-5), 24.2 (C-14), 15.2 (C-12), 11.8 (C-13); EIMS, m/z (%) : 218 (M^+, 48), 135 (100), 107 (98), 96 (64), 67 (34), 41 (27).

4.4. Microorganisms

A total of 6 bacteria (*Staphylococcus aureus* ATCC25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Shigella dysenteriae* ATCC 26131, *Shigella boydii* ATCC 9234, *Sarcina lutea* ATCC 28106 and three fungi *Candida albicans* ATCC 90028, *Aspergillus niger* ATCC 235561, *Saccharomyces cerevisiae* ATCC 60782 were used in the experiments. The microbial species maintained on agar slant at 4°C and sub-cultured on a fresh appropriate agar plate 24 prior to any antimicrobial test.

4.5. Culture media

Nutrient Agar was used for the disc diffusion method. Nutrient broth was used for the determination of MIC.

4.6. Disc diffusion method

The antimicrobial activity of the tested samples (extract, VLC fractions, zerumbone and reference drugs) were assayed by the standard disc diffusion method [17]. Tested samples were dissolved in methanol and injected into sterilized discs of 6 mm in diameter. Nutrient Agar media sterilized at 121°C by using the autoclave and cooled to 60–70 °C was distributed to sterilized Petri dishes with a diameter of 9 cm (15 mL). After preparation of the suspensions of test organisms (C.F.U 1.6×10^5 organisms per mL), 100µL of the suspension was added to each Petri dishes and distributed homogeneously. Dishes injected with tested materials were placed on the solid agar medium. Petri dishes were incubated at 37°C for 24 h. On each plate, an appropriate reference antibiotic (Amoxicillin 20 µg/disc and Nystatin 10µg /disc) disc and control (5µl methanol/disc) disc were applied. At the end of the period, inhibition zones formed on the NA were evaluated in millimeters. Studies were performed in triplicate, and the developing inhibition zones were compared with those of reference disc.

4.7. Minimum inhibitory concentration (MIC)determinations

The serial tube dilution technique was used to determine MIC of the compound against bacteria and fungi [18, 19]. Tested samples (2.048 mg) was dissolved in 2 mL distilled water (20 µL DMSO was used to make solution) to obtain the stock solution having a concentration of 1,024 µg/mL. In the serial dilution technique, 1 mL prepared stock solution was transferred to a test tube containing 1 mL nutrient broth medium to give a concentration of 512 µg/mL from which 1 mL was transferred to another test tube containing 1 mL nutrient broth medium to give a concentration of 256 µg/mL and so on up to a concentration of 2 µg/mL. After preparation of the suspensions of test organisms (1600 organisms per mL), 1 drop of suspension (0.015 mL) was added to each broth dilution. After 18 h incubation at 37 °C, the tubes were then examined for growth. The MIC of tested samples was taken as the lowest concentration that showed no growth. Growth was observed in those tubes where the concentration was below the inhibitory level and the broth medium was observed turbid (cloudy). Distilled water with 20 µL DMSO was used as negative control, while amoxicillin and nystatin were used as positive control.

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