Bangladesh Pharmaceutical Journal ***25****(1): 80-88, 2022 (January)*

Antibacterial Naphthaquinones from *Necterium* sp. Inhabited in *Tinospora cordifolia*

Gazi Monjur Murshid[1], Md. Hossain Sohrab[2], Mohammad Mehedi Masud[1] and Md. Abdul Mazid[1]

[1]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Dhaka Dhaka-1000, Bangladesh
[2]BCSIR Laboratories Dhaka, Dr. Qudrat-I-Khuda Road, Dhanmondi, Dhaka-1205, Bangladesh

Received: December 6, 2021; Accepted: January 25, 2022; Published (Web): January 29, 2022

Abstract

Numerous biologically active metabolites have previously been obtained from endophytes including endophytic fungi. This potentiality and prospect prompted us to find out new compounds having activity against pathogenic bacteria, from fungal endophyte, *Necterium* sp. isolated from the leaf of *Tinospora cordifolia*. We cultivated and developed the fungal strains on PDA medium and extracted on ethyl acetate after 28 days. Column chromatographic technique followed by preparative TLC was utilized to isolate secondary metabolites as pure crystalline solid. Two naphthoquinones namely anhydrofusarubin (**1**) and fusarubin methyl ether (**2**) were isolated from *Necterium* sp. The ^{1}H and ^{13}C NMR spectral data of the isolated compounds were analyzed and compared with previously published values to confirm the structure of the compounds. The compounds were assessed for antibacterial activity by resazurin based microdilution assay. Both of them showed sensitivity towards gram negative *Pseudomonas aeruginosa* but none was active against *Staphylococcus aureus*, a gram positive bacteria. The MIC values against *P. aeruginosa* for anydrofusarubin (**1**) and fusarubin methyl ether (**2**) were found to be 32- and 64-µg/ml respectively while the standard gentamycin showed MICs of 6.25 µg/ml (for *S. aureus)* and 12.5 µg/ml (for *P. aeruginosa*). The outcomes of our study suggest that anhydrofusarubin (**1**) and fusarubin methyl ether (**2**) are beneficial lead compounds to broaden the sphere of marketed antibacterial drugs.

Key words: Endophytic fungi; *Necterium* sp.; Antibacterial activity; Anhydrofusarubin; Fusarubin methyl ether.

Introduction

A fungus living inside the plant part as an endosymbiont is an endophytic fungus (Stone *et al*., 2000, Clay and Schardl, 2002). Numerous endophytic fungi have the potential to produce bioactive secondary metabolites to be utilized against a number of diseased conditions (Strobel *et al*., 2004; Staniek *et al*., 2008; Aly *et al*., 2010; Kharwar *et al*., 2011; Kusari and Spiteller, 2012). However, the need to search for new antibacterial agent is always getting priority to scientists. The development of resistance against infectious bacteria is a prime concern nowadays and endophytic fungi could pave the way to find out novel antibacterial agents. A lot of examples of secondary bioactive metabolites could be cited which have promising therapeutic values; few include hypericin and emodin (Kusari *et al*., 2008; Kusari *et al*., 2009c), podophyllotoxin (Eyberger *et al*., 2006; Puri *et al*., 2006), deoxypodophyllotoxin (Kusari *et al*., 2009a), azadirachtin (Kusari *et al*., 2012) and camptothecin as well as its synthetic analogs (Puri *et al*., 2005;

Corresponding author: Md. Abdul Mazid, Email: ma.mazid@du.ac.bd, Tel: +880-2-9661900 ext. 8131
DOI: https://doi.org/10.3329/bpj.v25i1.57844

Kusari *et al.*, 2009b; Shweta *et al.*, 2010; Kusari *et al.*, 2011). It is, therefore, evident from different studies that fungal endophytes yield diversified novel bioactive compounds of various structural backbones (Senadeera *et al.*, 2012; Wibowo *et al.*, 2014; Chokpaiboon *et al.*, 2016).

We targeted to investigate new antibacterial compounds and selected a prosperous medicinal plant *Tinospora cordifolia* (Local name: Gulancha, Family: *M*enispermaceae). Endophytic fungus, *Necterium* sp. was isolated from the leaf of this plant. Previously published reports show that few important metabolites have been isolated from *Necterium* sp. including newer cyclohexane derivatives like nectriatone A, nectriatone B and nectriatone C; phenolic sesquiterpene derivatives like ilicicolin D, ilicicolin E and deacetylchloronectrin (Yua *et al.*, 2018), heptaketides like pseudonectrin A, pseudonectrin B, pseudonectrin C, pseudonectrin D (Peinan *et al.*, 2019). However, very few works have done so far to isolate bioactive antibacterial compounds from *Necterium* sp. and therefore, we conducted this study on quest of bioactive antibacterial compounds from *Necterium* sp.

Ethyl acetate extract was collected after 28 days matured culture of endophytic fungus, *Necterium* sp., grown on PDA medium. We obtained two naphthoquinones (Figure 1) from the extract in pure form. Column chromatographic technique followed by preparative TLC was applied to obtain the compounds. Antibacterial activity was assessed by resazurin-based MIC assessment method. Isolated pure compounds showed prominent antibacterial activities.

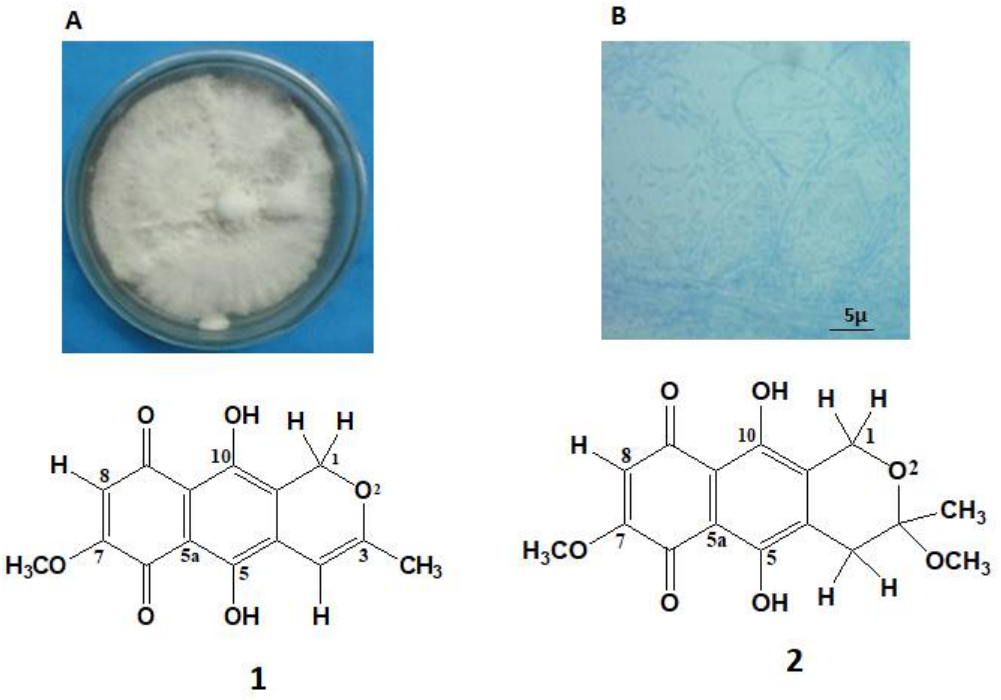

Figure 1. Chemical structures of anhydrofusarubin (1) and fusarubin methyl ether (2) isolated from *Necterium* sp. (A. Macroscopic view and B. Microscopic view 100X).

Materials and Methods

General experimental procedures: Isolated pure compounds were studied under nuclear magnetic resonance (NMR) spectrometer with a unity of 400 MHz; deuterated chloroform was used to dissolve the compounds. The ^{1}H and ^{13}C NMR spectral data were acquired and the structures of the compounds were elucidated by spectroscopic analysis as well as by comparing with previously published NMR data.

Isolation of secondary metabolites: Necterium species, internal strain no. TCLE-2, was isolated as per the standard procedure. Surface sterilization technique was performed by using sodium hypochlorite from the fresh leaf of the plant *T. cordifolia* to ensure inoculation of endophytic fungus only. The fungal strain was then cultivated at room temperature for 28 days on PDA medium. We extracted the culture medium with ethyl acetate after the fungus being matured; three times the extraction was performed keeping 7 days duration in between each extraction and 2.0 gm extract was obtained. Column chromatographic technique was utilized for fractionation over silica gel using gradients of petroleum ether-dichloromethane (DCM), then DCM, followed DCM-methanol and finally methanol to come up with a total of 21 fractions. The obtained fractions were screened by a series of TLC on silica gel under UV light in both short (254 nm) and long (365 nm) wavelengths. One of the column fractions obtained from petroleum ether-80% dichloromethane was subjected to preparative TLC using toluene-ethyl acetate (4.9: 0.1) for further fractionation. After elution with DCM then 1:1 DCM-EA followed by DCM with MeOH two pure compounds were obtained from two sub-fractions; fine needle shaped structure of 8 mg of compound **1** and 5 mg of compound **2.**

*Compound **1**:* The compound was observed on TLC plate as black-violet spot. It was found freely soluble in chloroform and DCM while in methanol it was sparingly soluble. Its R_f value was found to be 0.42 in toluene- EtOAc (95:5); ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.04 (3H, s, CH_3-3), 3.94 (3H, s, OCH_3-7), 5.25 (2H, s, CH_2-1), 6.03 (1H, s, H-4), 6.21 (1H, s, H-8), 12.69 (1H, s, OH-5), 13.08 (1H, s, OH-10); ^{13}C NMR (100 MHz, $CDCl_3$): δ 20.09 (C-11), 56.6 (C-12), 62.9 (C-1), 94.75 (C-4), 108.01 (C-9a), 109.9 (C-8), 111.0 (C-5a), 122.7 (C-10a), 133.1 (C-4a), 158.0 (C-10), 158.0 (C-5), 160.0 (C-7), 161.5 (C-3), 177.7 (C-6), 182.8 (C-9).

*Compound **2***: The compound was appeared on TLC plate as dark spot. The compound was found freely soluble in DCM and chloroform while in methanol it was sparingly soluble. Its R_f value was 0.44 in toluene-EtOAc (80.20); ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.57 (3H, s, CH_3- 11), 2.67 (1H, dt, $J_{4,4}$ = 18.0 Hz, $J_{4,1}$ = 2.0 Hz, H-4), 2.99 (1H, dd, $J_{4,4}$ = 18.0 Hz, $J_{4,1}$ = 1.5 Hz, CH_3-4), 3.34 (3H, s, OCH_3-12), 3.95 (3H, s, OCH_3-13), 4.57 (1H, dt, $J_{1,1}$ = 17.8 Hz, $J_{1,4}$ = 2.7 Hz, H-1), 4.88 (1H, dd, $J_{1,1}$ = 17.8 Hz, $J_{1,4}$= 1.5 Hz H-1), 6.20 (1H, s, H-8), 12.69 (1H, s, OH-5), 12.97 (1H, s, OH-10); ^{13}C NMR (100 MHz, $CDCl_3$):δ 22.8 (C-11), 33.0 (C-4), 48.9 (C-2), 56.7 (C-13), 58.7 (C-1), 96.8 (C-3), 107.5 (C-9a), 109.7 (C-5a), 109.9 (C-8), 132.9 (C-4a), 137.2 (C-10a), 157.2 (C-7), 160.6 (C-5), 160.9 (C-10), 184.6 (C-9).

Antibacterial assay: Broth micro-dilution was employed to assess the antimicrobial activity of compounds **1** and compound **2** along with few more compounds not isolated from this fungal strain. Two bacterial strains, namely gram positive *Staphylococcus aureus* (ATCC 25923) and gram negative *Pseudomonas aeruginosa* (ATCC 27853) were employed to serve the purpose. Pure cultures of bacterial strains were collected from the Biotechnology Discipline of Khulna University, Bangladesh. Broth micro-dilution method (Wiegand *et al.*, 2008) was used to determine the minimum inhibitory concentration (MIC), with slight modification. Firstly, sterilized agar medium was poured into petri dishes and kept for a few minutes to prepare agar slants. With an aseptic environment, we transferred the test organisms onto agar slants under laminar air flow. Inoculated slants were then hatched at 37°C for 16-18 hours to confirm the growth of test organisms. Secondly, the sterilized nutrient agar medium was poured into petri dishes and it was kept for few minutes to be solidified. In an aseptic condition we transferred the test organisms from the first culture to the nutrient agar slants. Incubation of test organisms was performed at 37°C for 24 hours to ensure the entrance in the growth phase. For each bacterial inoculum preparation, the bacterium from the second culture was taken by loop and transferred into a sterile capped glass tube containing sterile 0.9% NaCl and mixed properly using a vortex mixer to ensure growth on culture media. The turbidity of

inoculum was measured in a densitometer and the bacterial count was determined by comparing with the McFarland standard (we prepared 0.5 *McFarland* standard by mixing 9.95 ml of 1% sulfuric acid with 0.05 mL of 1.175% $BaCl_2.2H_20$). After turbidity adjustment, the bacterial suspensions were used within 30 minutes to ensure geometric integrity of microbes. We dispensed 100 µl of double strength nutrient broth into each well of 96-well sterile plates and added calculated volumes of the stock solutions of extracts to the appropriately labeled wells; the final concentrations of 256,128, 64, 32, 16 and 8 µg/ml were adjusted in a serial dilution manner. Then 5 µl of resazurin solution was added to each well (resazurin dye was dissolved in 2 ml of sterile water and mixed well to prepare resazurin solution). Prepared 96-well plate was covered and incubated for 18 to 24 hours at 37°C. The absorbance was taken by micro-titre plate reader at 570 nm wavelength. A change in color was observed visually. A blue or purple color of the well indicated the inhibition of growth. Gentamycin was used as the standard. Later, we calculated the percent inhibition for all concentrations as per the formula: 100 - (absorbance of sample/absorbance of positive control) x100, while positive control is the bacterial strain with broth only.

Results and Discussion

Cultivation in large scale for *Necterium* sp. on PDA medium was arranged followed by the extraction on ethyl acetate after 28 days. Two naphthoquinones, namely anhydrofusarubin (compound **1**) and fusarubin methyl ether (compound **2**) were obtained after chromatographic separation and fractionation by Preparatory TLC. Structural elucidation was performed by ^{1}H-and ^{13}C-NMR report and was confirmed by comparison with previously published data.

On visual observation, compound **1** was obtained as violet colored; purple spot was observed on TLC plate. The compound was isolated from a column fraction with the eluent system petroleum ether-DCM (20:80). The ^{13}C NMR spectrum (100 MHz, $CDCl_3$) of compound **1** displayed 15 carbon resonances. A methyl group resonance was observed at δ 2.04 ppm in the ^{1}H NMR (400 MHz, $CDCl_3$) and at 20.09 ppm in the ^{13}C NMR spectra. Presence of a methoxy group in compound 1 was confirmed by the resonance at 3.94 ppm in the ^{1}H NMR and at 56.6 ppm in the ^{13}C NMR spectra. Two-olefenic proton resonances were observed at 6.03 ppm and 6.21 ppm in the ^{1}H NMR spectrum while the corresponding carbons were seen at 94.7 and 109.9 ppm in the ^{13}C NMR spectrum. The presence of two equivalent aliphatic protons was indicated by singlet at 5.25 ppm in the ^{1}H NMR and at 62.9 ppm in the ^{13}C NMR spectra. Two chelated phenolic hydroxyl groups attached to C-5 and C-10 were evident with the occurrence of two downfield proton singlets at 12.69 and 13.08 ppm in ^{1}H NMR spectrum. Intramolecular hydrogen bonds with lone pair of electrons of a functional group could be constituted due to the relatively deshielded nature of these hydroxyl groups. Two carbonyl carbons were clearly indicated by the presence of two peaks at 177.7 ppm and 182.8 ppm in the ^{13}C NMR spectrum. A quinone system merged within the aromatic ring was indicated by two shielded carbonyl carbons with the fused aromatic ring. Three signals at δ 161.5, 158.0 and 158.0 in the ^{13}C NMR spectrum confirmed the attachment of three carbons to the hydroxyl and methoxyl groups. Another oxygen bearing carbon, C-3 was indicated by the presence of signal at δc 160.0. In compound **1**, it is apparent that two intramolecular hydrogen bonds were formed by the carbonyl groups of quinone system and two deshielded phenolic hydroxyl groups were also formed. The compound is evidently a derivative of oxy-anthraquinone derivative with one methoxyl, two hydroxyl and one methyl groups. Previously published NMR data of isolated anhydrofusarubin (James and Robert, 1983; Kurobane *et al*., 1986; Huque *et al*., 2016) from the fungus *Fusarium solani* aided us to confirm the structure of compound **1** as anhydrofusarubin.

Compound **2** was obtained as orange crystals on visual inspection from a sub-column fraction with petroleum ether-DCM (80%) followed by PTLC of the sub-fraction with 4.9:0.1 toluene-ethyl acetate. It

was appeared as orange spot on TLC plate. The ^{13}C NMR spectrum (100 MHz, $CDCl_3$) of compound **2** displayed 16 carbon resonances**.** The presence of a methyl group was ascertained due to the signal at δ 1.57 in the ^{1}H NMR (400 MHz, $CDCl_3$) and at 22.8 ppm in the ^{13}C NMR spectra. The appearance of two methoxyl groups in the compound 2 was evident with the resonances at δ 3.34 ppm and 3.95 in the ^{1}H NMR and at δ 48.9 ppm and 56.7 in the ^{13}C NMR spectra. Presence of one-olefenic proton was confirmed by the resonances at 6.20 ppm in the ^{1}H NMR and at 109.9 ppm in the ^{13}C NMR spectra. Two aliphatic protons were manifested with the occurrence of one one-proton doublet of triplet at 4.57 ppm and another one proton doublet of doublet at 4.88 ppm in the ^{1}H NMR spectrum. Two chelated phenolic hydroxyl groups were evident with appearance of two intense proton singlets at 12.69 and 12.97 ppm in ^{1}H NMR spectrum. These apparently deshielded hydroxyl groups might be responsible to constitute intramolecular hydrogen bonds with any lone pair electrons of a functional group. The presence of carbonyl group in the structure was confirmed by the appearance of resonance at 184.6 ppm in the ^{13}C NMR. A quinone system merged to the aromatic ring was confirmed by fairly shielded nature of the carbonyl carbons. In compound **2** two intramolecular hydrogen bonds with the two deshielded phenolic hydroxyl groups constituted the carbonyl groups of quinone system. The structure of compound **2** was elucidated as fusarubin methyl ether and confirmed by comparing these spectral data with the published NMR values of fusarubin methyl ether reported from the fungus *Fusarium solani* (James and Robert, 1983; Kurobane *et al.*, 1986; Huque *et al.*, 2016).

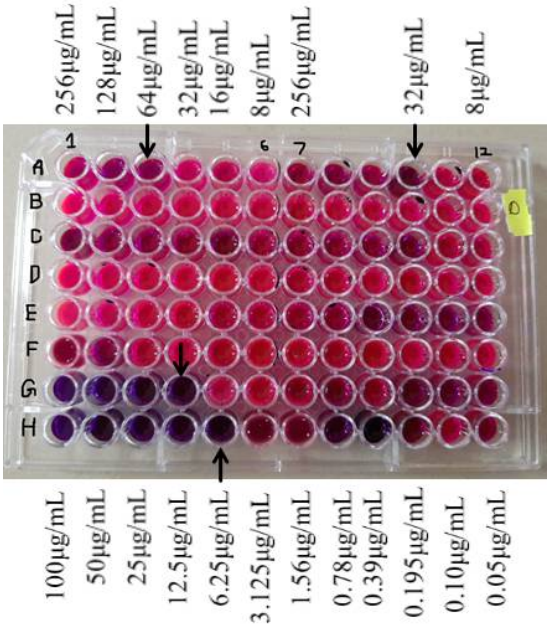

Figure 2. MIC visualized by very first discoloration on resazurin (Arrow-marked): compound **1**- Anhydrofurasubin (AHF), MIC on A10=32 µg/ml and compound **2-** Fusarubin methyl ether (FME) on A3=64 µg/ml both against *P. aeruginosa*; Gentamycin (std.) on G4 = 12.5 µg/ml against *P. aeruginosa* and H5 = 6.25 µg/ml against *S. aureus.* Here, **A (1-6):** Fusarubin methyl ether against *P. aeruginosa*, **A (7-12):** Anhydrofusarubin against *P. aeruginosa*; **B (1-6):** Fusarubin methyl ether against *S. aureus*, **B (7-12):** Anhydrofusarubin against *S. aureus*; C (1-6): Phytol against *P. aeruginosa*, C (7-12): Eugenol against *P. aeruginosa*; D (1-6): Phytol against *S. aureus*, D (7-12): Eugenol against *S. aureus;* E (1-6): Thymol against *P. aeruginosa*, E (7-12): CAF against *P. aeruginosa*; F (1-6): Thymol against *S. aureus*, F (7-12): CAF against *S. aureus;* G (1-12): Gentamycin (std) against *P. aeruginosa*, H (1-12): Gentamycin against *S. aureus*. Concentration used on column wells: A1-F1 & A7-F7: 256 µg/ml, A2-F2 & A8-F8: 128 µg/ml, A3-F3 & A9-F9: 64 µg/ml, A4-F4 & A10-F10: 32 µg/ml, A5-F5 & A11-F11: 16 µg/ml, A6-F6 & A12-F12: 8 µg/ml; For G1-G12 & H1-H12 concentrations used were 100 µg/ml to 0.05 µg/ml as per serial dilution.

The isolated pure compounds **1** and **2** along with few more compounds (not isolated from this fungal strain) were evaluated for antibacterial activities by resazurin based MIC assay using broth dilution method. Two pathogenic bacterial strains, namely, gram positive *S. aureus* and gram negative *P. aeruginosa* were used for the study. Both Compound **1** (anhydrofusarubin) and compound **2** (fusarubin methyl ether) showed activity against *P. aeruginosa*. Compound **1** and compound **2** showed activity against *P. aeruginosa* with a visible MIC at the concentrations of 32 µg/ml and 64 µg/ml, respectively while the MIC value of standard gentamycin was 12.5 µg/ml for the same (figure 2). Later the percent inhibition value (50%) were calculated for all concentrations against *P. aeruginosa* using the formula: 100-(absorbance of sample/absorbance of positive control) x 100. 50 percent of inhibition was observed at 32 µg/ml and 16 µg/ml for fusarubin methyl ether and anhydrofurarubin, respectively (Table 1) while for standard gentamycin it was found on 0.19 µg/ml (Table 2). Methicillin-resistant gram-positive strain *S. aureus* was found unresponsive or resistant to both the compounds in the present study. Usually *S. aureus* is known as notorious for its ability to become resistant to antibiotics. Few reasons include its hydrolyzing ability to β-lactam ring of antibiotics, excessive production of β-lactamase leading to quick binding with extracellular antibiotics followed by the inactivation of the drug (Khoshnood *et al*., 2018), formation of biofilm (Kanwar *et al*., 2017; Craft *et al*., 2019; Saxena *et al*., 2019), acquisition of resistance genes (Lazaris *et al*., 2017) and so on. For example, *S. aureus* is known to form penicillinase and this enzyme can disrupt the β-lactam ring of penicillin. This results in penicillin resistance of this bacterial strain. Even 2 years after methicillin being developed on 1959 methicillin resistant *S. aureus* (MRSA) became rapidly the frequent resistant pathogens worldwide (Lakhundi and Zhang, 2018). All these factors altogether made the gram-positive *S. aureus* a tough candidate to combat with and it could explain rationally why this bacterial strain was found resistant to our compounds.

Table 1. Calculated percent inhibition values for anhydrofusarubin (AHF) and fusarubin methyl ether (FME).

Concentration (µg/ml)	FME	AHF
8	45.38±0.27	46.19±0.50
16	46.47±0.15	**53.53±0.15**
32	**51.63±0.28**	57.88±0.41
64	62.77±0.15	60.59±0.47
128	64.95±0.41	63.58±0.15
256	68.75±0.27	67.66±0.41

Table 2. Calculated percent inhibition values for standard Gentamycin against *P. aeruginosa.*

Concentration (µg/ml)	Gentamycin (Std.)
0.05	45.38±0.15
0.1	46.20±0.15
0.19	**51.63±0.27**
0.39	63.59±0.27
0.78	66.03±0.41
1.56	68.21±0.27
3.12	69.02±0.15
6.25	73.37±0.15
12.5	75.82±0.15
25.0	77.99±0.41
50.0	79.89±0.15
100.0	81.52±0.27

Our finding indicates that *Necterium* sp. resides within *T. cordifolia* produces bioactive naphthoquinones having useful antibacterial property. It is in line with the previous reports since a number of compounds like napthaquinones and aza-anthraquinones display activity against microbes with précised mechanism of action (Marumo *et al*., 1980). Among the two isolated compounds anticancer activity of anhydrofusarubin was previously reported; however, very few reports are available on its

putative antibacterial effect. In a previous study, anhydrofusarubin was screened for action against a number of gram positive and gram negative pathogenic bacteria, yeasts and fungi; it was found to be responsive to most of the pathogens in different degrees (Ammar *et al*., 1979). Other study report revealed that compounds including fusarubin and 3-O-methyl fusarubin isolated from *Fusarium solani* fungal strain exhibited sensitivity towards the tested bacterial strains (Shah *et al.,* 2017). Anhydro-fusarubin and fusarubin methyl ether both are derivatives of fusarubin, hence, we assumed earlier to have the putative antibacterial effect from these compounds. In another experiment the researchers studied the mode of antimicrobial naphthoquinones isolated from the fungus *Fusarium sp.* by using *P. aeruginosa*; fusarubin and fusarubin methyl ether both were found to stimulate the oxygen consumption of bacterial cells explaining their possible antibacterial mechanism (Haraguchi *et al*., 1997). From our study result we found the activity of fusarubin methyl ether and anhydrofusarubin against *P. aeruginosa* which supports all the previous findings. Antibacterial effect of fusarubin methyl ether was also determined previously (Huque *et al*., 2016) by disc diffusion method; however, broth microdilution (used in the present study) is a more précised quantitative method to draw conclusion on susceptibility against microbes (Kim and Kim, 2007). Still our study is a preliminary one and further studies are required on diversified bacterial strains with more extensive studies, *i.e.*, the method of broth diffusion, antibiofilm test, time-kill study and other recognized studies to quantify the extent and intensity of antibacterial effect.

Naphthoquine and its derivatives are reported to exert antibacterial activity by plasmid curing/inactivation, interference with the activity of efflux pump and production of reactive oxygen species in bacteria (Mone *et al.*, 2021). A study conducted on a series of synthesized naphthoquinone derivatives concluded that the mechanism of antibacterial effect is related to bacterial membrane binding (Ravichandiran *et al*., 2019). Another study revealed that naphthoquinones initiated an augmented level of lipid peroxidation in *S. aureus* and initiated toxic or lethal effect to the bacterial cells (Medina *et al*., 2006). Naphthoquinones being less potent than other conventional antibiotics could exert a synergistic effect along with the antibiotics if treated against MRSA infections (Yap *et al*., 2021). Evidences are also there that naphthoquinones act as antibacterial agent by inhibiting DNA gyrase (Karkare *et al*., 2013). The mechanism of antibacterial action of anhydrofusarubin and fusarubin methyl ether is obviously related to one or more of the aforementioned mechanisms. Our plan is to find out the mechanism of action of antibacterial activity of anhydrofusarubin and fusarubin methyl ether in the next phase of experiment.

Conclusion

We report the antibacterial activity of pure compounds isolated from *Necterium* species residing within the plant *T. cordifolia*, first time in Bangladesh. The present work has offered two secondary metabolites from the fungal strain *Necterium* sp., which were characterized as anhydrofusarubin (**1**) and fusarubin methyl ether (**2**) and both of them were found to have antibacterial potentiality. A further study is recommended by the researchers.

Acknowledgements

This study was funded by the University Grants Commission (UGC) of Bangladesh (No. 37-01-0000-073-04-006/2019/2483). Pharmaceutical Sciences Research Division and Chemical Research Division of BCSIR Laboratories, Dhaka provided necessary research facilities to carry out the study. The first author is thankful to the Ministry of Science & Technology, Government of the People's Republic of Bangladesh to provide Bangabandhu Research Fellowship.

Conflict of Interest

The authors declare no conflicts of interest regarding the content of the manuscript.

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