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Isolation and biological evaluation of thiazole ethanol derivative secondary metabolite from marine derived fungus Aspergilliusterreus

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ABSTRACT

Marine derived products have been promising sources for the detection of unique bioactive compounds and gaining importance for their biotechnological applications. Aspergillus terrus was isolated from the marine sample by serial dilution and the novel basal media formulated in this study resulted in the synthesis of the fungal secondary metabolite. The structure of the bioactive secondary metabolite was analyzed by ^{1}H-, ^{13}C-NMR and mass spectral data as 1-(4,6-dihydro-2-vinylfuro[3,4-d] thiazol-6-yl)ethane-1,2-diol. Different concentrations of the compound (250,500,750 & 1000µg/ml) were prepared and tested against the test microorganisms using well diffusion method showed strong inhibitory activities. IC_{50} value of the compound was 1.24mg/ml, suggesting a strong antioxidant activity and the DPPH radical scavenging ability of the compound was significantly lower when compared to that of ascorbic acid.

Keywords: Marine; Thiozole; Aspergillusterrus; Secondary metabolites; DPPH

1. INTRODUCTION

Fungi from marine sources have served as rich sources of new biological and natural products. Owing to particular living conditions, salinity, nutrition, high pressure, temperature variations, competition with bacteria, viruses and other fungi, they may have attained specific secondary metabolic pathways when compared to the terrestrial fungi [11]. Recently it is fascinating to know The diversity of secondary metabolites . Genera, such as Aspergillus is known for their ability to produce various chemical compounds known as metabolites which have been revived making screening program less efficient. The physical and biological variations in natural environment favor the yield of a diverse range of secondary metabolites [7].

*A. terreus*is occasionally reported as a pathogen of human and animals and the compounds present possess various pharmacological and commercial values. *A. terreus*is producer of secondary metabolites. Compounds that are produced by *A. terreus*are aspulvinone [15], asterric acid [6], asterriquinone [9], citrinin [14], emodin [4], geodin [10], itaconate [3], quercetrin [6] and sulochrin [16]. Lovastatin, an antihyperlipidemic drug is used in the treatment of heart disease and atherosclerosis. Apart from bioactive compounds, A. terreus has also produced some mycotoxins such as Citreovividin and terretonin. The present study has dealt with the

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investigation of structurally unique and active secondary metabolites of Aspergilliusterreus obtained from marine source.

2. MATERIALS AND METHODS

Sample source

Marine sea water was collected from the Bay of Bengal in Chennai, (Marina beach, Tamilnadu) at 1-2m depth, stored below 4°C and processed within 24 hrs.

Isolation of fungi

Isolation of fungus was done by following the methods of Choi et al., (1999) [5] and Ho et al., (2001) [8]. Serial dilution of seawater was done until 10-6 and pre incubated at room temperature for 1hr for the activating the dormant cells. The aliquot was plated on Czapekdox agar media with 5% sodium chloride and incubated at room temperature (27±2ºC) for ten days. The morphologically distinct fungal spores were then isolated by successive subculturing on Czapekdox agar media.

Fermentation and isolation

The fungus cultured on Czapekdox agar (Hi-media) plates was kept for 7 days at 25°C. The mycelial disks (8 mm in diameter) was transferred into 2L Erlenmeyer flask consisting of 800 ml of minimal media containing 2.0mg Tryptophan, 4.088g K2Hpo4, 0.72g Thiamine hypochloride, 0.780g Glycine, 0.768g Mgso4 and4.32g Lactose supplemented with 100 mg/L streptomycin (Sigma) thereby preventing the growth of bacteria and incubated for 21 days at 26 °C in a rotary shaker (180 rpm). The extraction of filtrate from the culture was done thrice with ethyl acetate and dried over anhydrous sodium sulfate. Evaporation was done to yield the crude extract (651mg). Activated silica gel (60–120 mesh) was packed onto a glass column (65 mm × 8 mm) using n-hexane solvent and 651mg of crude ethyl acetate extract was further loaded on the top of the silica gel column. The column was eluted with the mixture of hexane and ethyl acetate and the fraction containing an intense spot was focused to isolate the pure compound. The pure fraction was subjected to HPLC on an analytical reverse C18 phase column (150mm×4.6 mm, 0.5µm) using a mobile phase acetonitrile and water (60:40) for 30 minutes with a flow rate of 0.5 ml/minute to confirm the purity. 1H NMR spectrum was recorded on Bruker (500 MHz, MEOD) with tetramethylsilane (TMS) as the reference, 13C NMR (100 MHz, MEOD) and the chemical shifts were expressed in δ ppm.

Determination of antibacterial activity

The antibacterial activity of metabolite was tested against *Staphylococcus aureus*and *Pseudomonas aeroginosa, Proteus mirabilis, Klebsiellapneumoniae* and *E. coli* by well diffusion method [12]. Different concentrations of the extract (250, 500, 750 and1000 µg/mL) were

prepared by dissolving the test compound in dimethyl sulfoxide (DMSO) and were added to appropriate wells. The control and the petriplates treated wells were incubated at 37 ℃ for 24 h. The zone of inhibition (mm) were measured. Positive control was Streptomycin and the solvent was the negative control.

DPPH radical scavenging activity

Radical scavenging activity was performed following the method of Blois (1958) [2]. The antioxidant capacity was expressed as the antioxidant activity index (AAI) and calculated as: AAI = final concentration of DPPH (mg·mL-1)/IC50 (mg·mL-1). The AAI was calculated after taking into consideration the mass of DPPH and the mass of the tested compound in the reaction. In general, the AAI of the tested compound is classified as a poor antioxidant activity when AAI < 0.5, moderate antioxidant activity when the AAI is between 0.5 and 1.0 and strong antioxidant when the AAI is between 1.0 and 2.0, and very strong when AAI > 2.0 [1].

Molecular docking studies for predicting antioxidant and antibacterial activities

To bolster the assay studies with the mechanism of binding, molecular docking study was carried out for the marine compound isolated from Aspergilliusterreus. Drug target proteins such as src family kinase (2HCK) of SH3-like barrel fold, which falls into Hemapoetic cell kinase Hck domain with enzyme classification of transferase protein kinase activity was taken as the antioxidant drug target and the dithiol oxidase (DsbA) enzyme role as disulfide oxidoreductase activity for biological process of cell redox homeostatsis and oxidation-reduction process from Proteus mirabilis is an attractive drug target for antibacterial activity. Dithiol oxidase (DsbA) enzyme has a role in the virulence pathogenesis of gram negative organisms (4OD7). These two drug targets were initially prepared by removing bound ligands, cofactors, ions, identical chains, and water molecules and were then subjected to the energy minimization and protein preparation to remove bad steric interactions and alternative conformers. On contrary, the isolated ligand structures were converted from the chemical format to PDB (Protein Data Bank). Thus, using MGL tools 1.5.6, protein and ligand were prepared for auto grid and auto dock. Before simulations, gasteiger charges were computed for both the drug targets and the ligand root, torsion angles were calculated and saved in docking format, followed by auto grid calculation for atom types with grid center 36.635X 41.003Y 130.861Z and -34.801X 24.814Y 22.356Z for 2HCK and 4OD7, respectively. Consequently, the free energy binding was calculated by the Lamarckian genetic algorithm (LGA), finer than the other docking methods present in auto dock.

3. RESULTS AND DISCUSSION

Isolation and identification of secondary metabolites

Fungus was isolated from the marine sample by serial dilution; the fungus emerged out for the number of times was considered as a dominant fungus and it was successfully sub cultured in Czapekdox agar. For the morpho cultural characterization, the marine fungus isolated was

initially cultured on Czapekdox agar plates without antibiotics and the inoculated culture plates were incubated at room temperature for 24 – 48 h. For studying cell morphology, the isolated fungus was stained with phenol cotton blue for 1 min. These stained cells were observed and measured under microscope (400-1,000x, Olympus CX31)for determination of the shape and size . The isolate was identified as marine Aspergillusterreus by its morphology. The fungus consisted of branch, multinucleate, septate hyphae and conidial head. The marine A.terreus was subjected to secondary metabolite production in an optimized minimal medium. The mycelium and culture filtrate (800 mL) were extracted with ethyl acetate and evaporated under vacuum at 45 °C to obtain a white residue (651mg). In this study, the fraction containing an intense spot produced by A.terreus was focused to isolate pure compound. Fractionation of the crude extract of A.terreus was subjected to column chromatography. Homogeneity of fractions on TLC plates were combined and concentrated together to give the pure compound. The 800 mL fermentation broth was yield 47.4 mg of isolated compound. Compound 1 was isolated as a light yellow liquid [α] D23 at 250C +22.4 (c=0.5, MeOH), UV (MeOH), λmax: 220 nm. Its molecular formula (C9H11O3SN) was deduced based on the EI MS ion at m/z 210 (calc for [m+H+] 212, [m-2] 211), indicating four degrees of unsaturation. The 1H NMR spectrum indicated the presence of a terminal double bond at 3.23 δppm (m, J=7.2Hz) and 7.66(t, J=3.2Hz), along with an epoxy ring system with 8.18 and 4.15 δppm. The secondary and tertiary alcohols contributed to the signals at 5.2, 6.2, 3.82 and 3.63 δppm. Nine carbon signals in the 13C NMR spectra were assigned to the two epoxy ring carbons (57.10 & 60.83 δc) and the two hydroxyl bearing carbons (65.99 & 69.91δc). The signals at the downfield region indicated the presence of a double bond with two peaks (122.76 & 126.94 δc), two signals for unsaturation (147.21 and 150.21 δc) followed by a prominent peak at 165.17δc indicative of a carbon attached to the S and N atoms. The IR spectrum of the compound also inferred the presence of C=N, and -OH stretching. Based on the above findings, the structure of 1 was determined to be 1-(4,6-dihydro-2-vinylfuro[3,4-d]thiazol-6-yl)ethane-1,2-diol (Fig-1), which according to Sci-finder reports was designated as a unique secondary metabolite from the marine fungus.

Antibacterial activity

The antimicrobial activity is shown in table-2. The compound showed a strong antibacterial activity against the gram negative bacterium i.e. Pseudomonas aeroginosa with a inhibition zone of 31mm at the concentration of 1000µg/mL. The inhibition zone varied depending on the concentration. Compared to antibiotics, the compound showed exhibited activity against all the pathogens. The inhibitory effect of the compound decreased in the order against: P.aeroginosa>E.coli> S. aureus> K. pneumonia > P. mirabilis at the concentration of 1000µg/mL.

DPPH radical scavenging activity

Activity of the compound is shown in Fig 2. It is concentration dependent and reached the maximum at 2.0 mg/mL. The compound exhibited a strong antioxidant property, which was

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clearly evident by its low IC50 value of 1.24 mg/mL. The ability of the compound was significantly lower than ascorbic acid as 0.08mg/ml, the Antioxidant Activity Index (AAI) was 1.61, suggesting a strong antioxidant potential and for ascorbic acid, the well-known strong antioxidant, AAI was found to be 6.25 [13].

Molecular docking

Molecular docking is an in silico study dealing with the theoretical investigation of the biological assay; hence, it is considered as a boon to the biological research to study the atomic level interactions and binding of the compound with the protein active site. Autodock is one of the best softwares to study protein-ligand interactions. In genetic algorithm, 20 runs with 150 population size, 27000 and 2500000 maximum number of energy and generations and the top-ranked lowest binding energy run in the histogram chart was chosen for the interaction analysis (Figure 3). 2HCK-ligand formed strong hydrogen and hydrophobic interactions (Table 3) similar to 4OD7-ligand forming hydrogen, hydrophobic and other interactions with the active site aminoacid (Table 1). The estimated free energy of binding of 2HCK (-6.52 Kcal/mol) had a Ki value 16.69 uM while 4OD7 energy values (-4.83 Kcal/mol) were less than 2HCK with Ki of 287.64 uM as the estimated inhibition constant. Hence, the least energy value and Ki value indicated the biological activity of the compound in in vivo studies. Likewise, SAR studies were conducted for this compound replacing Sulphur(S) as the methylcarbon, which showed less interactions and the least negative free energy binding, further extensively. Nitrogen replacement of the methyl group affected the overall free energy binding for both the drug targets. Thus, from the perspective of SAR, these two groups were considered as a crucial pharmacophore of this molecule. Even though shape is a complement to the active site, the functional pharmacophore is important for bioefficacy of the compound.

Figure. 1 Structure of the isolated compound 1-(2-vinyl-4,6-dihydrofuro[3,4-d]thiazol-6-yl)ethane-1,2-diol

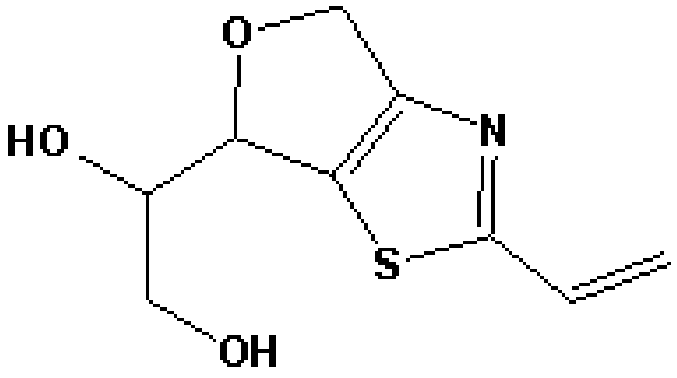

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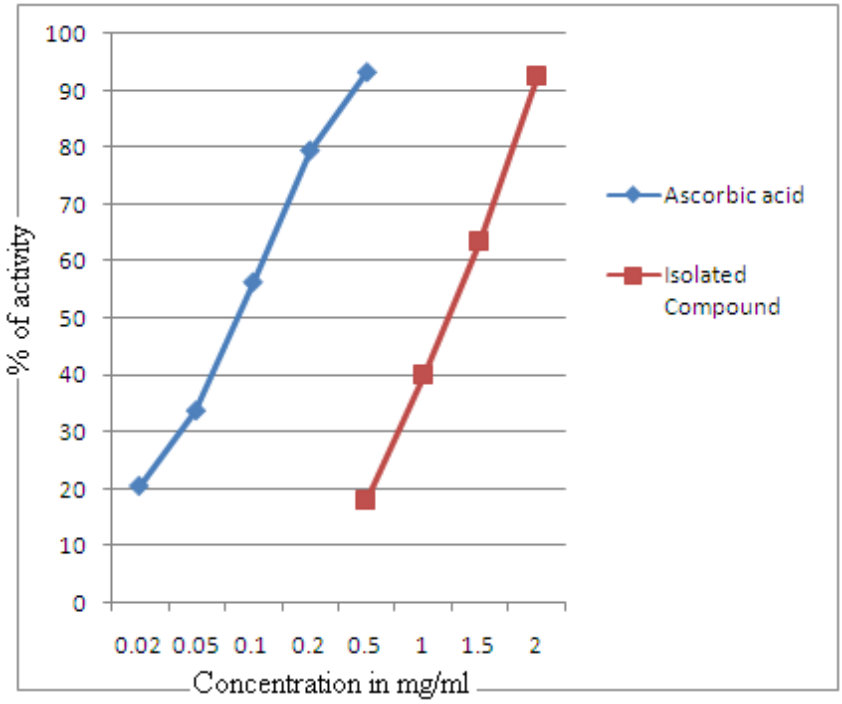
Figure. 2 DPPH radical scavenging activity



Values are expressed in mean, n=3.

Figure. 3 2HCK active site amino acid and ligand binding interaction (A, B); 4OD7 active site amino acid and ligand binding interaction (C, D)

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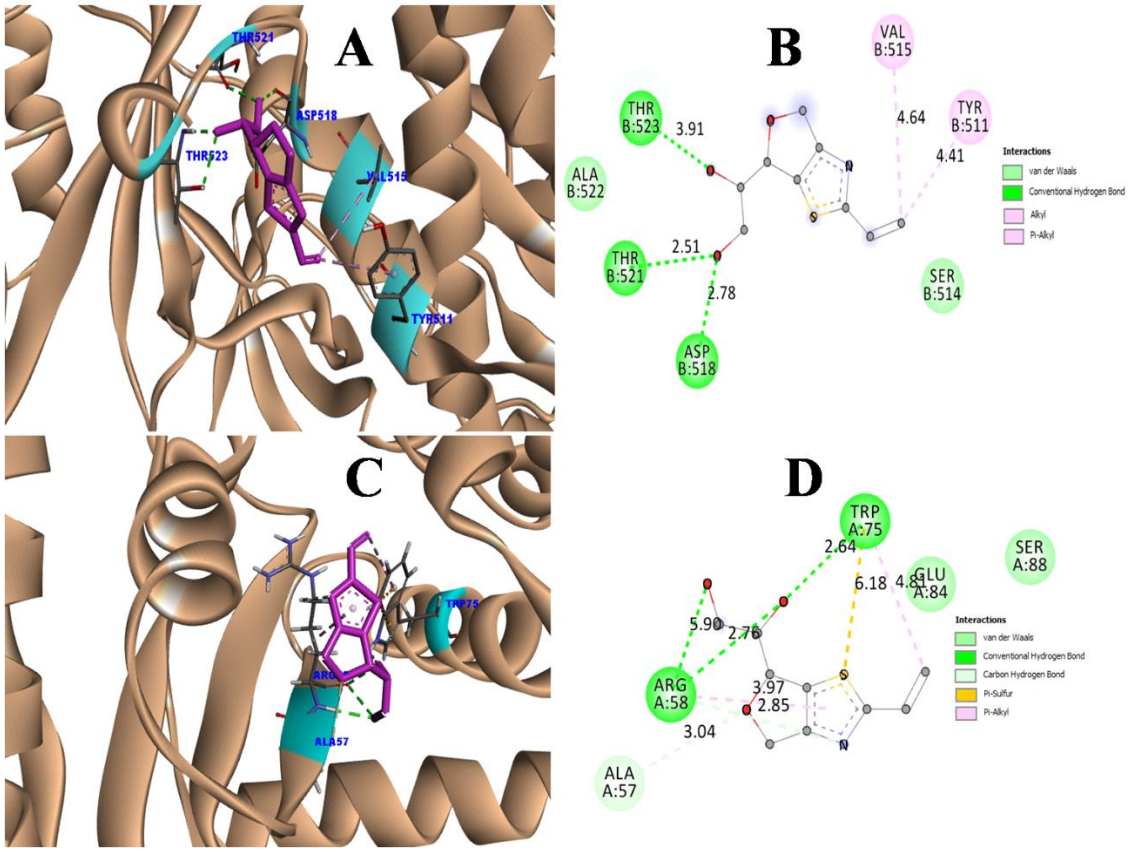

Table-1 Spectroscopic data for the isolated compound

^{13}C NMR (δppm)	**^{1}H NMR (δppm)**	**Assignment**	**MASS(m/z)**	**IR cm^{-1}**
165.17	-	Carbon attached to imine N and Thiol group	(M+) 213 Fragments: (M-2) 211,	3925, 2940, 1543, 1825, 1040, 986, 745
150.21, 126.94	-	Unsaturation on furan ring		
147.21 122.76	8.1 (t,1H), 7.6 (d, 2H)	Terminal Olefinic bond		
69.91	5.17 (d, 1H)	Furan carbon bearing ethane diol side chain		
65.99,60.83, 57.10	4.15 (t,1H), 3.83 (m,2H), 3.64 (m,2H)	Carbons attached to O-		
-	3.32 (s,3H)	Alcohol protons		

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Table- 2- Antibacterial activity of the isolated compound

Organisms	Inhibition zone in mm							
	250 µg/mL		500 µg/mL		750 µg/mL		1000 µg/mL	
	A	C	A	C	A	C	A	C
Escherichia coli	15.2 ± 0.2	22.2 ± 0.1	18.2 ± 0.1	20.1 ± 0.05	20.2 ± 0.1	24.2 ± 0.1	22.1 ± 0.2	28.0 ± 0.1
Pseudomonas aeroginosa	24.1 ± 0.1	25.1 ± 0.05	26.2 ± 0.1	27.1 ± 0.1	28.3 ± 0.1	30.3 ± 0.1	30.2 ± 0.1	31.0 ± 0.05
Proteus mirabilis	14.3 ± 0.1	15.1 ± 0.1	18.1 ± 0.1	18.2 ± 0.2	20.1 ± 0.1	19.9 ± 0.1	25.0 ± 0.1	22.0 ± 0.05
Klebsiellapneumoniae	14.2 ± 0.1	12.2 ± 0.1	17.1 ± 0.05	14.2 ± 0.1	20.2 ± 0.2	19.0 ± 0.1	23.1 ± 0.1	23.2 ± 0.1
Staphylococcus aureus	20.2 ± 0.2	16.1 ± 0.1	24.1 ± 0.1	13.2 ± 0.05	27.2 ± 0.05	22.1 ± 0.1	29.0 ± 0.1	27.1 ± 0.1

Values are expressed in mean ± SD, n=3.
A-Isolated compound; C-Positive control

Table 3: Receptor-ligand interaction of the drug target-ligand complex

Drug target-Ligand complex	Atom-atom Interaction	Distance in Å	Category
2HCK-Ligand	B:THR523:HN - :UNK0:O3	2.05383	Hydrogen Bond
	B:THR523:HG1 - :UNK0:O3	2.4034	Hydrogen Bond
	:UNK0:O4 - B:ASP518:O	2.78228	Hydrogen Bond
	:UNK0:O4 - B:THR521:O	2.51093	Hydrogen Bond
	:UNK0:C14 - B:VAL515	4.63844	Hydrophobic
	B:TYR511 - :UNK0:C14	4.40791	Hydrophobic
4OD7-Ligand	A:ARG58:HN - :UNK0:O4	2.11027	Hydrogen Bond
	A:TRP75:HE1 - :UNK0:O3	2.64303	Hydrogen Bond
	:UNK0:O3 - A:ARG58:O	2.75827	Hydrogen Bond
	:UNK0:O4 - A:ARG58:O	2.7576	Hydrogen Bond
	A:ALA57:HA - :UNK0:O10	3.03997	Hydrogen

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			Bond
	A:ARG58:HD1 - :UNK0:N7	2.85291	Hydrogen Bond
	:UNK0:S5 - A:TRP75	5.73788	Other
	:UNK0:S5 - A:TRP75	4.55663	Other
	A:TRP75 - :UNK0:C14	4.80632	Hydrophobic
	:UNK0 - A:ARG58	3.97483	Hydrophobic

4. CONCLUSION

The secondary metabolites have strong biological activities. These products are used in therapy, produced by microbial fermentation, or from chemical alteration of a microbial product. The present study of screening bioactive secondary metabolite revealed *A.terreus* as a source for the synthesis of novel bioactive metabolite can be further worked upon for biotechnological applications in medicine and agriculture.

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