In vitro T-cell inhibition of Aurasperone –A from a marine derived fungus *Aspergillus fonsaceus*

M H Haroon[1,2,3]* , S R Premaratne[1], M I Choudhry[3] and H R W Dharmaratne[1]

[1]Natural Products Programme, Institute of Fundamental Studies, Kandy

[2]Faculty of Applied Sciences, South Eastern University of Sri Lanka, Oluvil.

[3]H.E.J Research Institute of Chemistry, University of Karachi, Pakistan

haroonmh@seu.ac.lk

Abstract

Aurasperone –A (**1**) was isolated from the culture broth of a fungus *Aspergillus fonsaceus,* which was separated from a brown alga along with Adenine (**2**), Uridine (**3**) and Phenyl acetic acid (**4**). Structures of compounds **1**-**4** were deduced by the spectroscopic data. Compound **1** showed a potential suppressive effect on T-cell proliferation inhibition assay.

Keywords: Aurasperone –A, *Aspergillus fonsaceus,* Adenine, Uridine, Phenyl acetic acid, T-cell proliferation inhibition assay.

1. Introduction:

Marine derived endophytic microorganisms represent a rich source of biologically active metabolites [1]. The identification and characterization of novel T-cell inhibitory compounds is important for developing new strategies for the prevention and treatment of autoimmune and allergy disorders. In particular, treatments that are safe, well-tolerated, and capable of suppressing T-cell activation processes or modulating the balance of Th1/Th2 subsets are especially promising [2]. T-cell activation plays a crucial role in the initiation and regulation of the immune response. Activation of T-lymphocytes is a complex process. It is characterized by secretion of the lymphokine interlekin-2 (IL-2) and by expression of the membrane receptor for IL-2 on the T-cell. Stimulation of this receptor by the secreted IL-2 leads to proliferation. T-cell proliferation is estimated by measuring [H] thyamidine incorporation [3]. Therefore, Inhibition of T-cell activation provides powerful approach for immunosuppressive treatments [3].

In our continuing studies on biologically active metabolites from marine derived fungi [4], we examined the chemistry of *Aspergillus fonsaceus,* isolated from a marine brown alga *Sargassum wightii* collected from Tangalle, South coast of Sri Lanka, gave a Aurasperone –A (**1**), along with Adenine (**1**), Uridine

ISBN 978-955-627-120-1

(**2**) and Phenyl acetic acid (**3**). Compound **1** has been isolated from *Aspergillus tubingensis* [5]. It is unlikely that these molecules are isolated in a free state with conventional isolation procedure as they are usually occur as polymeric forms in nature. Adenine and uridine are precursor molecules for genetic material in living organisms and commonly known as nucleosides.

2. Experimental:

2.1. General experimental procedures

Melting points were determined with a Gallenkamp hot stage apparatus (Cat No. MPD 350.BM 2.5). The observed melting points were uncorrected and given in centigrade units. The ^{1}H NMR spectra were recorded in CD_3OD on AMX-500 NMR spectrometers at 500 MHz. The ^{13}C NMR experiments were conducted on the same instrument at 125 MHz. HREI MS and FAB MS were carried out on Jeol JMS HX 110 mass spectrometer. Chemical shifts were given in δ_{ppm} units (s-singlet; d-doublet; t-triplet, m-multiplet), relative to the chemical shift of tetramethylsilane (TMS), as an internal standard and coupling constants *J* in Hz. Analytical TLC was carried out with 0.2 mm thick silica gel (60 F 254) pre-coated Aluminum sheets.

2.2. Seaweed material

Marine algal/seaweed sample, *Sargassum wightii* investigated in this study was collected from, Tangalla (Southern coastal area) in Sri Lanka and identified by comparison with herbarium specimens at the Royal Botanic Gardens, Peradeniya, Sri Lanka. A herbarium specimen of this sample is deposited at the Natural Products Chemistry laboratory, Institute of Fundamental Studies, Kandy, Sri Lanka by fixing in salt water buffered, 3-5% formalin.

2.3. Fungal material and fermentation

The fungus *Aspergillus fonsaceus,* was separated from *Sargassum wightii*, a brown alga, collected at Tangalle; Southern coastal area in Sri Lanka. Fungal cultures were sub cultured on commercially available Malt Extract Agar (MEA-Oxoid CM 59) to observe morphological characters. Cotton blue was used as the dye. Slide culture technique was used to characterize the fungal cultures. According to the morphological data, this fungus was identified as *Aspergillus*

ISBN 978-955-627-120-1

fonsaceus. The endophytic fungus was isolated from the blades of the seaweeds using the method developed to isolate endophytes from higher plants by Petrini [6]. The fungus was grown in Czapek Dox liquid medium containing 3% peptone ($NaNO_3$, 1.5 g; KH_2PO_4, 0.5 g; $MgSO_4$. $7H_2O$, 0.25 g; KCl, 0.25 g; $FeSO_4$.$7H_2O$, 0.005 g; Sucrose,15 g; Peptone, 15 g; Natural Sea Water, 250 mL; Distilled Water, 250 mL) for 14 days at 28 ^{0}C. The cultured broth (13 L) was filtered.

2.4. Extraction and separation

The filtrate of the cultured broth (13L) was extracted with EtOAc (13 L x 2). The EtOAc was evaporated using rotary evaporator at 50 ^{0}C. The EtOAc extract was dried in a vacuum oven to afford a brown residue (1.5 g). This EtOAc extract was subjected to column chromatography (CC) on flash silica gel (Merk 60, 230-400 mesh) to yield 4 compounds. This column was initially eluted with gradient polarities of n- hexane and EtOAc, which afforded 4 fractions. (F -1 to F-4)
Repeated silica gel (SiO_2) column chromatography of F-1 (5% EtOAc: hexane) yielded pure compound **4** (4.2 mg). Fraction F-2, when subjected to flash column chromatography, yielded pure compound **1** (6 mg, 15%Acetone: hexane). Repeated C_{18} reverse phase column chromatography of F-3 gave pure compound **2 (**3 mg, 5% MeOH: H_2O**)** while fraction F-4 yielded pure compound **3** (3 mg , 6% MeOH: H_2O)

Aurasperone –A (**1**)
Dark yellow colored powder (5 mg); mp.290-291 ^{0}C.
^{1}H NMR (500 MHz, CD_3OD) δ: 2.15 (3H, s, Me), 6.08 (1H, s, H-3), 3.97 (3H, s, OMe), 6.41 (1H, s, H-7), 3.59 (3H, s, OMe), 6.50 (1H, s, H-9), 2.41 (3H, s, H-2'), 6.14 (1H, s, H-3'), 3.48 (3H, s, OMe), 3.80 (3H, s, OMe), 6.23 (1H, s, H-9').
^{13} C NMR (100 MHz, CD_3OD) δ: 20.6 (CH_3-3), 107.8 (C-3), 56.6 (OMe), 111.2 (C-7), 55.6 (OMe), 98.0 (C-9), 20.8 (CH_3), 107.8 (C-3'), 62.4 (-OMe), 56.6 (-OMe), 97.5 (C-9'), 103.0 (C-10); $C_{32}H_{26}O_{10}$, EI MS (70 eV) *m/z* (rel.int.%): 570 (100.), 285 (75)

Adenine (**2**)
White amorphous solid (3 mg); mp.360-365 ^{0}C.
^{1}H NMR (300 MHz, DMSO-d6) δ; 2.5 (2H, s, NH_2), 7.01 (1H, s, H-3), 12 (1H, broad, NH), 8.15 (1H,d, H-7); $C_5H_5N_5$, EI MS (m/z 135.0, $[M]^+$).

ISBN 978-955-627-120-1

Uridine (**3**)
Colorless crystals (3 g); mp.165-170 ^{0}C.
^{1}H NMR (300 MHz, CD_3OD) δ: 4.6 (1H,s, NH), 5.90 (1H, d, H-5), 7.95 (1H,d, H-6), 5.70 (1H, d, H-1'), 3.85 (1H,dd, H-2'), 3.75 (1H,dd, H-3'), 4.15 (1H, m, H-4'), 4.0 (1H,dd, H-5'), 4.18 (1H, dd, H-5'); $C_9H_{12}N_2O_6$, EI MS (m/*z* 244 $[M]^+$).

Phenyl acetic acid (**4**)
Pale yellow crystals with a characteristic bee honey smell (4.2 mg); mp.75-79 ^{0}C.
^{1}H NMR (300 MHz, $CDCl_3$) δ: 9.6 (1H, s, COOH), 3.7 (2H, s, H-1), 7.3 (1H, d, H-3), 7.35 (1H, d, H-4), 7.32 (1H, d, H-5), 7.35 (1H, d, H-6), 7.3 (1H, d, H-7); $C_8H_8O_2$, EI MS (m/z 136, $[M]^+$).

2.5. *T-Cell Proliferation Inhibition Assay:*

2.5.1. *Isolation of Peripheral mononuclear Cells (PBMCs) from Human:*

A blood sample of (20 mL) was collected in the heparin containing tube from a healthy volunteer. Then, the blood, Ficoll paque (LSM, Histopaque-1077, Sigma, St. Louis, MO)
and RPMI (1640 Sigma) incomplete media were added in equal volume in to the 50 mL of sterile falcon tube aseptically. This mixture was layered (without mixing) on three sterilized centrifuge tube containing 5 mL Ficoll paque (LSM) and they were centrifuged at 400 x g for 20 minutes at room temperature. After centrifugation, the peripheral blood mononuclear cell (PBMCs) was appeared at the junction of the two layers and it was transferred into another sterilized tube and washed with RPMI incomplete media and centrifuged it again at 300 x g for 10 minutes at 4 ^{0}C. The pellet was resuspend in the 1 mL of RPMI incomplete media and the viability of cells were checked by trypan blue and counted by Hemocytometer [7].

2.5.2. T-cell proliferation:

50 μL of 5% complete RPMI was added into each well of a sterilized 96 well round bottom plate in sterile environment using safety cabinet and then the samples were added into the each well and diluted to get 3-different concentration.50 μL of PBMC cells (1x 10^6 cells/mL) in suspension of 5% complete RPMI was added to each well except blank and then 50 μL of PHA was added to each well except negative control and blank (5% complete RPMI without compound was used as control). Then, the each well volume up to 0.2

ISBN 978-955-627-120-1

mL by using 5% complete RPMI media and the mixture was incubated for 72 hours in CO_2 incubator at 37 ^{0}C. After the incubation, 25 µL of $[H]^3$-Thamidine was added to each well except blank and incubated again the plate in CO_2 incubator at 37 ^{0}C for 18 hours. The plate was removed from incubator and the cells were harvested using glass fiber filter and washed with 70% ethanol 5 times and kept filters until become dry. Finally, the filters which contain cells were dissolved by using scintillation solution and reading was taken by scintillation counter [8].

3. Results and Discussion:

The ethyl acetate extract of *Aspergillus fonsaceus* was subjected to repeated column chromatography on silica gel to yield Aurasperone –A (**1**), Adenine (**2**), Uridine (**3**) and Phenyl acetic acid (**4**). The EI-MS of Compound **1** showed an $[M]^+$ at 570 corresponding to the molecular formula $C_{32}H_{26}O_{10.}$ The EI MS peak at m/z 570 along with 285 indicated the presence of two rubrofusarin [5] units in the compound **1**. The ^{1}H-NMR spectrum of **1** showed signals due to 4 aromatic protons, 2 olephinic protons, 4 methoxy groups and 2 methyl groups. A pair of meta coupled protons (J = 2.7 Hz) at δ 6.41 and δ 6.50 and two methoxy groups at δ 3.97 and δ 3.59 further confirmed that Aurasperone –A has rubrofusarin (**2**) as part of its structure. Among the ^{1}H-NMR signals, those at δ 2.15 (CH_3-2) and 6.08 (H-3) in conjugation with HMBC and HMQC were readily associated with β-methyl-γ- pyrone group. In the ^{1}H-NMR spectrum, the long range coupling were observed at δ 2.15 (CH_3-2, d, J = 1.2 Hz) and δ 6.08 (H-3, m) and also at δ 6.41 (H-3', m) and δ 2.41 (CH_3-2', d, J = 1.3 Hz). Further this coupling were also observed in COSY spectrum. On the basis of the above spectroscopic evidences, the structure of compound **1** was deduced as Aurasperone –A which has been isolated from *Aspergillus tubingensis*[5].

ISBN 978-955-627-120-1

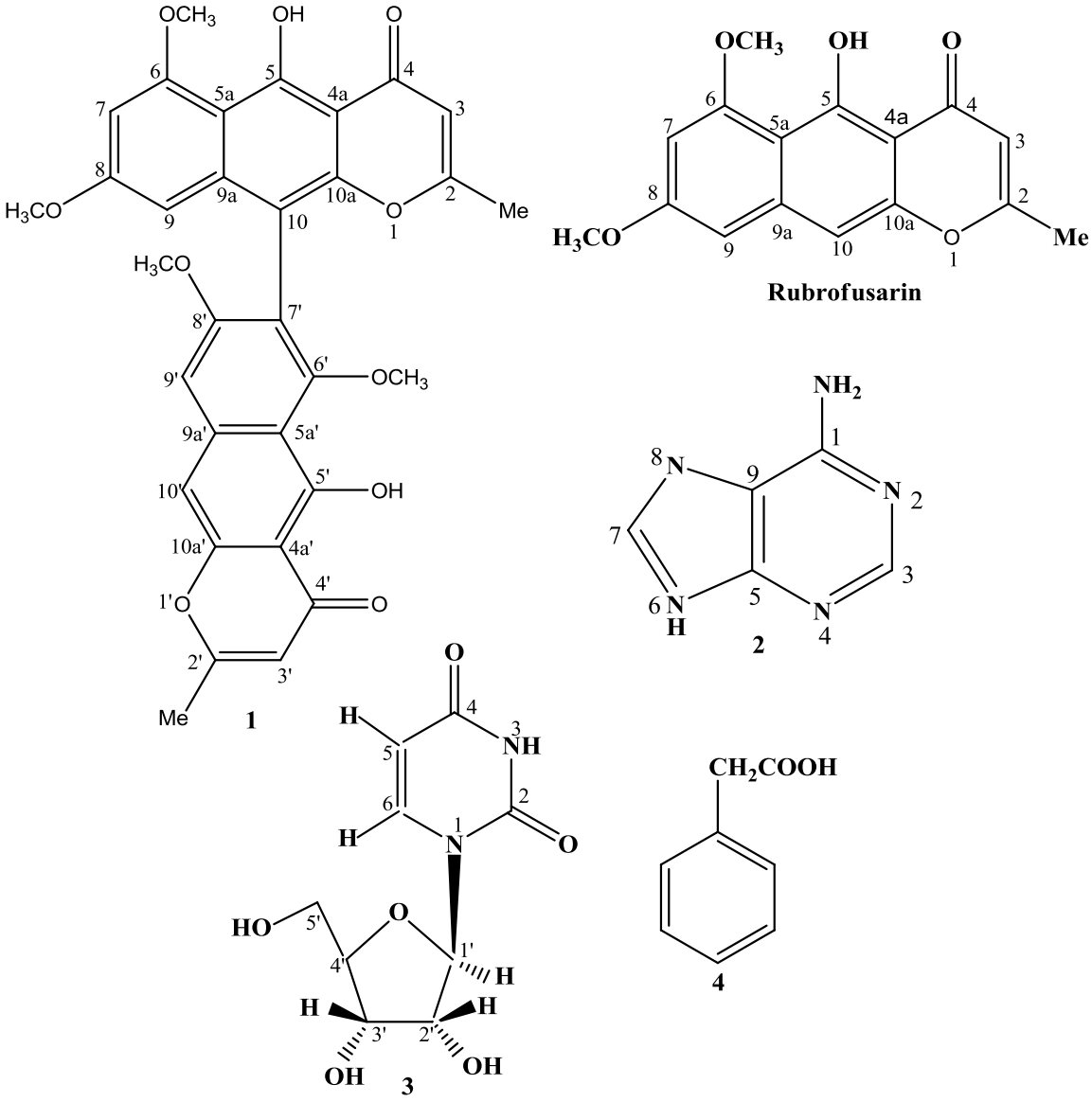

Fig. 1. Structures of Compounds 1— 4

In addition to the compound **1**, the structures of known compounds **2-4** were identified through comparison of their spectroscopic data. Compound **1** showed a potent anti proliferative effect (Figure-2) on PHA-induced T-cell proliferation assay with the IC_{50} value of <3.12 µg/ml compared to the standard Prednisolone (IC_{50} = 0.197 ± 0.02 µg/ml), clinically used drug for control inflammatory and allergic conditions.

ISBN 978-955-627-128-1

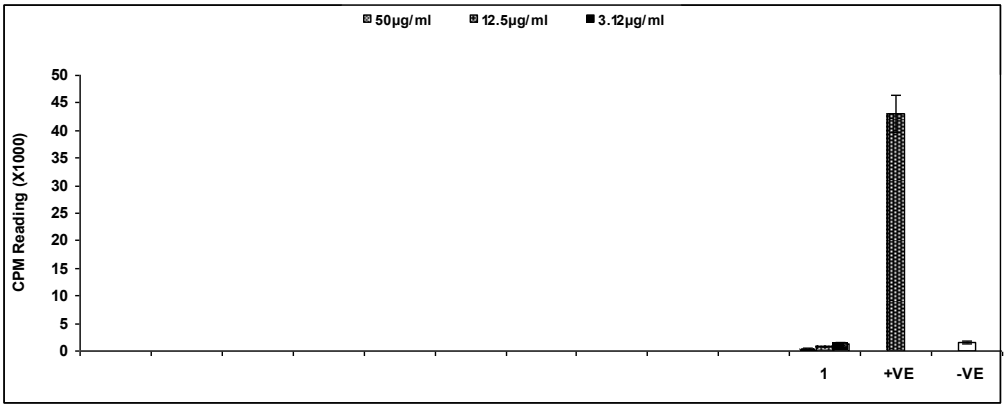

Fig.2: Effect of Aurasperone on T-Cell Proliferation assay

Conclusion:

Results showed that Aurasperone –A showed a potential suppressive effect on T-cell proliferation inhibition assay. It could be used as a candidate for the treatment of inflammatory diseases.

Acknowledgement:

The authors express their gratitude to Director, of the National Herbarium, Royal Botanic Gardens, Peradeniya for assisting with identification of brown alga. Institute for Organic and Biomolecular Chemistry, George-August University, Gottingen, Germany is greatly acknowledged for spectroscopic data.

References:

Strobel, G. A., *Microbes and Infections*, 2003, 5, 535-544.

Olson, R.D., Headley, M.B., Hodzic, A., Walsh, M.G., Wingett, W.G.,

Cerqueira, F.A., Cordeiro-Da-Silva, Araujo, N., Cidade, H., Kijjoa, A., Nascimento, M.S.J., *Life Sciences.* **2003**, *73,* 2321–2334

Haroon M H, S R Premaratne, M I Choudhry and H R W Dharmaratne..
Proceedings of Sri Lanka Association for the Advancement of Science, *64th annual session,* 2008, *Part 1, Abstracts* pp 151.

Asperpyrone D and Other Metabolites of the Plant-Associated Fungal Strain Aspergillus tubingensis, J. Zhan, G.M.K.B. Gunaherath, E.M.K. Wijeratne, and A.A.L. Gunatilaka, Phytochemistry, 2007, 68, 368-372

Petrini, O., *Taxonomy of endophytic fungi of aerial plant tissue*, 1991*, In* Verma, A., Abbott, L., Werner, D., Hampp, R., *Plant Surface Microbiology*. Springer-Verlag Berlin Heidelberg, New York, 2004.

Cerqueira, F.A., Cordeiro-Da-Silva, Araujo, N., Cidade, H., Kijjoa, A., Nascimento, M.S.J., *Life Sciences.* **2003**, *73,* 2321–2334

Repetto, M.G., and. Llesuy, F.G., *Brazilian Journal of Medical and Biological Research* **2002**, *35*, 523-534.

ISBN 978-955-627-128-1