Symbiotic Fungus of Marine Sponge Axinella sp. Producing Antibacterial Agent

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Abstract. The emerging of multidrug resistance pathogenic bacteria cause the treatment of the diseases have become ineffective. There for, invention of a new drug with novel mode of action is an essential for curing the disease caused by an MDR pathogen. Marine fungi is prolific source of bioactive compound that has not been well explored. This study aim to obtain the marine sponges-associated fungus that producing anti-MDR bacteria substaces. We collected the sponge from Riung water, NTT, Indonesia. The fungus was isolated with affixed method, followed with purification with streak method. The overlay and disk diffusion agar methods were applied for bioactivity test for the isolate and the extract, respectively. Molecular analysis was employed for identification of the isolate. The sponge was identified based on morphological and spicular analysis. The ovelay test showed that the isolate KN15-3 active against the MDR *Staphylococcus aureus* and *Eschericia coli*. The extract of the cultured KN15-3 was also inhibited the *S. aureus* and *E. coli* with inhibition zone 2.95 mm and 4.13 mm, respectively. Based on the molecular analysis, the fungus was identified as *Aspergillus sydowii*. While the sponge was identified as *Axinella* sp.

Keywords: Fungi, sponge, *Aspergillus sy*dowii, multidrug resistace, *Axinella* sp.

1. Introduction

Marine sponge is a source of bioactive compounds the pharmaceutical properties such as antioxidants [1], antibacterial [2], antifungal [3], anti-inflammatory [4,5], anti-malarial [6], and anticancer [7].

However, only few of them passed to the preclinical and clinical stages [8]. Compounds supply is a major problem for drug development from marine source [9]. One of the most serious bottlenecks in developing natural products from coral reefs has been the availability of biomass to gain sufficient amounts of substances for preclinical and clinical studies. Exploitation is further complicated by the fact that most of these metabolites possess highly complex structures, making them difficult to be produced economically via chemical synthesis. Sea culture is one alternative that may be used to solve the need of bioactive compounds from sponge though the sponge grows slowly [10,11]. Burgess *et al.* [12],

mentioned that bacteria symbiotically associated with soft corals can synthesize secondary metabolites similiar to host.

Sponge is a filter feeder animals so that microorganisms accumulate in large quantities as mesophyll. Some researchers believe that microorganisms play an important role, such as providing food, bioactive compounds, or precursors to their host [13]. Association number of bacteria in the sponge more than fungal symbionts, but fungal symbionts produce clinically active compounds is more important than bacteria [14].

This paper describes the isolation and identification of the associate fungus of marine sponge collected from Riung Water, East Nusa Tenggara, Indonesia.

2. Materials and methods

2.1. Sample collection

Marine sponges were collected from the Tujuh Belas Pulau, Riung, East Nusa Tenggara, Indonesia by SCUBA diving at 3-15 m depth. The specimens were kept in a cool box until inoculation process [15].

2.2. Isolation of fungi

Isolation of fungi on a sponge conducted with modification methods of Subramani *et al*. [16]. The sponges were washed with sterilized sea water prior to inoculation process to removed loosed associated microorganisms from its surface. Then, the specimen was cut into small piece (1 x 1 x 1 cm). The specimen laid on the surface of malt agar medium. After 7 days incubation, the fungus colonies were separated based on their morphological characteristics, and each colony was inoculated on a new agar plate contain MEA.

2.3. Antibacterial screening

Isolates of MDR bacteria, *Staphylococcus aureus* and *Escherichia coli*, was obtained from the Laboratory of Microbiology, Kariadi Hospital, Semarang. Culture of each MDR bacterium in the logarithmic phase was mixed with Zobell soft agar medium (1% v/v), which were then poured on to the respective agar surface previously inoculated with fungi symbionts sponge and incubated for 24 h [17]. The inhition zone indicated that isolates were active against the antagonistic bacteria (the data were not shown in this paper).

2.4. Sponge Identification

Identification sponge symbiont host of fungal isolates that produce antibacterial MDR *E. coli* and *S. aureus* based on morphological characteristics and the characteristics of spicules based methods of Hooper [18], with slight modifications. Morphological identification is done by color, surface shape, and the shape of growth.

Identification of the spicules is done by cutting a small fragment of the sponge and put in eppendorf microtub, then sodium hypochlorite was added. After the organic components were dissolved and leaving only the mineral skeleton, the bleach was diluted with ethanol and replaced carefully. The washing process was repeated several time. Spicules were taken and placed on a glass slide, and then observed under a microscope. the observations were documented by camera.

2.5. Extraction

The pure isolated shown active against *S. aureus* and *E. coli* were cultured on 1 L MEB media for 7 days or until the maximum growth at ambien temperature. The media was filtered to obtain the micellium that was extracted with methanol at room temperature. The solvent was filtered using filter paper and then dried with rotary evaporator [19].

2.6. Bioassay methanolic extracts

Bioassay methanolic extracts based on the method proposed by Safaeian *et al.* [20] with slight modification The extracts were tested againts the *S. aureus* and *E. coli* using difussion agar method at the concentrations 400, 200, 100, 50 and 25 µg/disk with two times repeated. The pathogenic bacteria were inoculated by spread method on MEA media, after 30 minutes incubation, the paper disks contained the methanolic extract were placed on the agar media surface.

2.7. Fungi KN-15-3 identification

2.7.1. Morphology identification

Fungi symbionts aged 7 days taken by ose needle and placed on a glass slide that has been etched with a solution of lactic acid then observed under a microscope with a magnification 40x. The results are documented using camera [21].

2.7.2. Molecular identification

The DNA isolate was extracted by *Wizard Genomic DNA purification Kit* following the instruction provided by the company (PROMEGA).

DNA engine thermal cycler (MyCycler, Biorad), PCR run comprised 34 cycle with cycle: initial denaturation at 95 ^{0}C for 5 minutes, annealing (57,1 ^{0}C for 1 min), extension (72 ^{0}C for 7 min), denaturation (94 ^{0}C for 40sec), and 42 ^{0}C for 1 min, 70 ^{0}C for 5 min, and the last 4 ^{0}C.

The DNA in supernatant was extracted with isopropanol for further analyses with electrophoresis on agrose gel. Electrophoresis was run at 100 volt with TAE (Tris-Acetate-EDTA) as running buffer. Ethadium bromida was utilized as pigment prior to visualization under UV light. The primers used for PCR amplification of 18S rRNA is universal primers ITS 1 (5'TCCGTAGGTGAACCTGCGG-3') and primer ITS 4 (5'TCCTCCGCTTATTGTATGC-3') [22]. Sequencing process was conducted in Macrogen, Korea.

3. Result and Discussion

From Riung Water of Nusa Tenggara Timur Province-Indonesia, a total of 18 sponges were collected that provided 33 fungus isolates. After antagonistic bioassay, the most active isolate, KN-15-3, was chosen for further study (the screening process was not performed in this paper).

The host of the isolate was also be identified base on morphological characteristics and spiculas analyses. Sponges host KN-15-3 has typical characteristicssuch such as in natural environment has a bright orange red color, while in alcohol brown or light brown, corrugated, with vertical or horizontal and branches in bush-shaped (Figure 1).

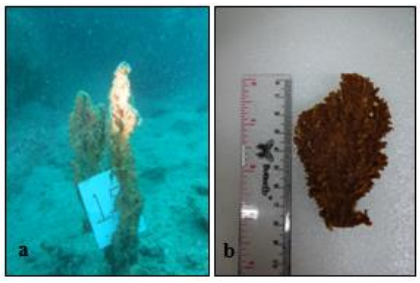


Figure 1. The photograph of the sponge K-N-15 (a). In situ (b). in the laboratory.

The identification of host sponge spicules KN-15-3 have type: style, subtylostyle, and strongyle (Figure 2). Based on the characteristics identification and spicules, sponges included in Genus Axinella [18, 23]. Genus Axinella known to be rich bioactive compounds such as anti-virus, cytotoxic, neurological activity, antioxidants and antifungals [24-28].

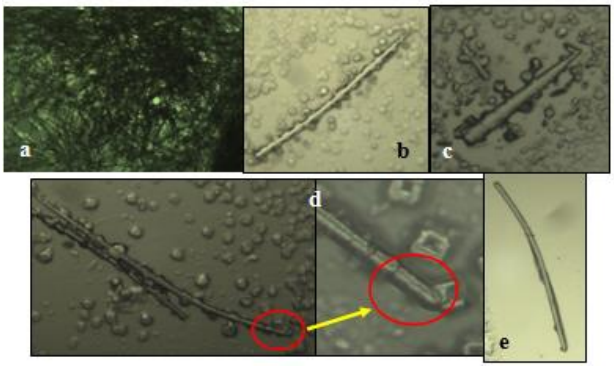


Figure 2. Spicules of the sponge K-N-15 (a = 10x, b-e = 40x) a= skeleton; b.,c. = Style; d = subtylostyle; e.= strongyle

On Malt extract agar media, fungal isolates have white mycelium and spores darker in color. On the observation by a microscope isolates KN-15-3, spores are round and surrounded by conidia thus forming like a flower (Figure 3).

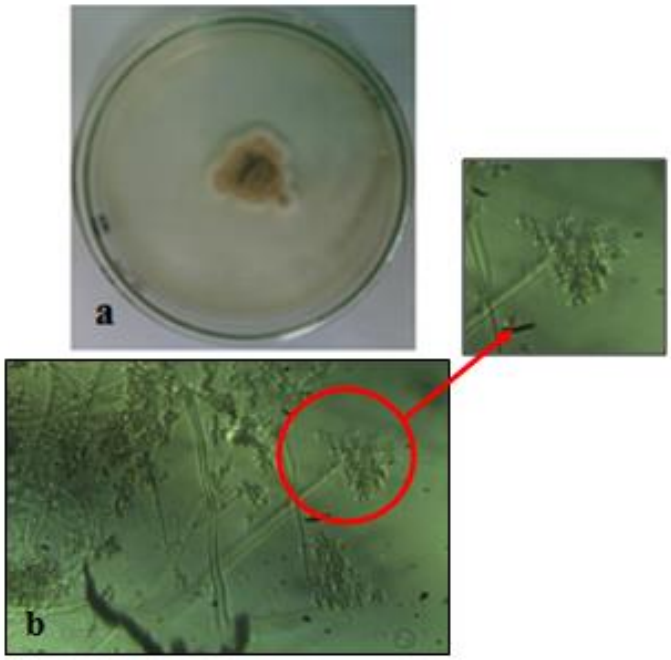
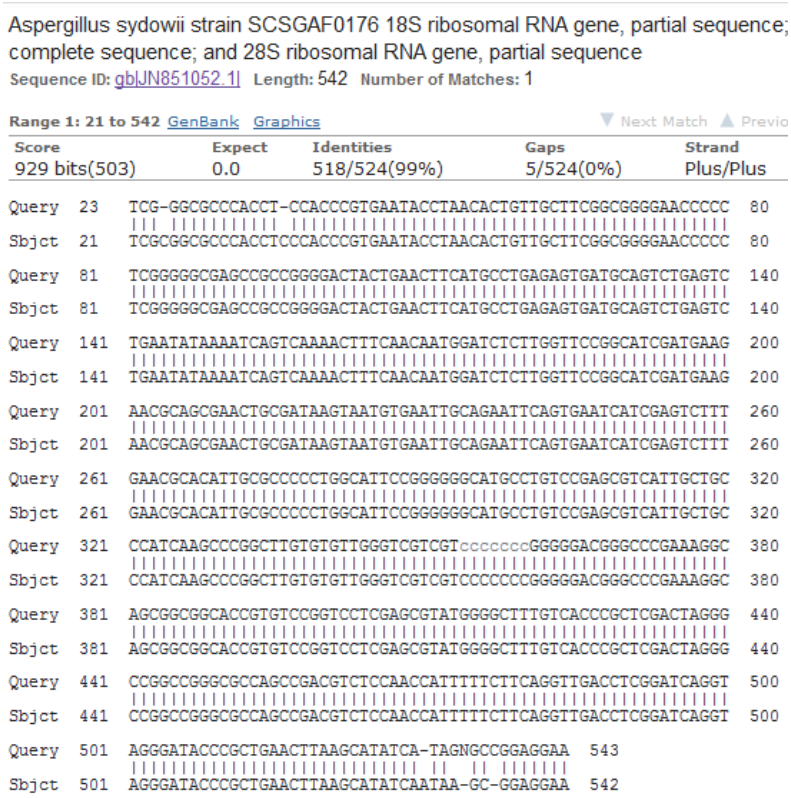


Figure 3. a. Fungi symbionts K-N-15-1. b. Mycelium of fungi symbiont sponge K-N-15-1

Based on the sequencing of fungal symbionts KN-15-3 has 99% similarity with the fungus Aspergillus sydowii (GenBank accession no. JN851052.1) (Figure 4). The isolate has been registered to GenBank with accession number LC094427.


Aspergillus sydowii strain SCSGAF0176 18S ribosomal RNA gene, partial sequence; complete sequence; and 28S ribosomal RNA gene, partial sequence

Sequence ID: gb|JN851052.1| Length: 542 Number of Matches: 1

Range 1: 21 to 542 GenBank Graphics Next Match Previo

Score	Expect	Identities	Gaps	Strand
929 bits(503)	0.0	518/524(99%)	5/524(0%)	Plus/Plus

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Query  23   TCG-GGCGCCCACCT-CCACCCGTGAATACCTAACACTGTTGCTTCGGCGGGGAACCCCC  80
            ||| |||||||||||| ||||||||||||||||||||||||||||||||||||||||||
Sbjct  21   TCGCGGCGCCCACCTCCCACCCGTGAATACCTAACACTGTTGCTTCGGCGGGGAACCCCC  80

Query  81   TCGGGGGCGAGCCGCCGGGGACTACTGAACTTCATGCCTGAGAGTGATGCAGTCTGAGTC  140
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  81   TCGGGGGCGAGCCGCCGGGGACTACTGAACTTCATGCCTGAGAGTGATGCAGTCTGAGTC  140

Query  141  TGAATATAAAATCAGTCAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAG  200
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  141  TGAATATAAAATCAGTCAAAACTTTCAACAATGGATCTCTTGGTTCCGGCATCGATGAAG  200

Query  201  AACGCAGCGAACTGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTT  260
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  201  AACGCAGCGAACTGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTT  260

Query  261  GAACGCACATTGCGCCCCCTGGCATTCCGGGGGGGCATGCCTGTCCGAGCGTCATTGCTGC  320
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  261  GAACGCACATTGCGCCCCCTGGCATTCCGGGGGGGCATGCCTGTCCGAGCGTCATTGCTGC  320

Query  321  CCATCAAGCCCGGCTTGTGTGTTGGGTCGTCGTccccccGGGGGACGGGCCCGAAAGGC  380
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  321  CCATCAAGCCCGGCTTGTGTGTTGGGTCGTCGTCCCCCCCCGGGGGACGGGCCCGAAAGGC  380

Query  381  AGCGGCGGCACCGTGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACCCGCTCGACTAGGG  440
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  381  AGCGGCGGCACCGTGTCCGGTCCTCGAGCGTATGGGGCTTTGTCACCCGCTCGACTAGGG  440

Query  441  CCGGCCGGGCGCCAGCCGACGTCTCCAACCATTTTTCTTCAGGTTGACCTCGGATCAGGT  500
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  441  CCGGCCGGGCGCCAGCCGACGTCTCCAACCATTTTTCTTCAGGTTGACCTCGGATCAGGT  500

Query  501  AGGGATACCCGCTGAACTTAAGCATATCA-TAGNGCCGGAGGAA  543
            ||||||||||||||||||||||||||||| ||  || |||||||
Sbjct  501  AGGGATACCCGCTGAACTTAAGCATATCAATAA-GC-GGAGGAA  542
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Figure 4. Results of sequence homology analysis isolates KN-15-3using the BLAST database.

The genus Aspergillus is the macroscopic filamentous fungi and one of the causes contaminants in food. This is caused by Aspergillus grows colonize and reproduce mycotoxins [29]. Aflatoxins are secondary metabolites produced by *A. flavus*, A. *parasiticus*, *A. nomius,* dan *A. tamarii* [29-32]. Aflatoxins can be found in many types of food and feed ingredients such as wheat, wheat flour, maize, cereals, rice, beans, spices, and beer [33-34].

The genus of Aspergillus is also known and recognized as a source of bioactive compounds in pharmacological field. Some bioactive compounds produced by Aspergillus genus such as antibacterial varixanthone, anticancer asperazine, antiparasitic gliotoxin 6-methoxyspirotryprostatin B, and antifungal Amphotericin B [35-38].

A.sydowii known to have some activity such as tyrosinase inhibitors, anthraquinone, as antimicrobial, sydowiols A and sydowiols C as anti *M. tuberculosis*, and as acetylcholinesterase inhibitor [39, 40]. Acetylcholinesterase inhibition resulted in increased concentrations of acetylcholine. This further result in increased communication between nerve cells that use acetylcholine as a chemical messenger producing a therapeutic effect in patients with Alzheimer's disease

In antagonistic test the isolate KN-15-3 showed active against two MDR bacteria *Staphylococus aures* and *Escherichia coli.* The methanolic extract of the isolate also exhibited strong activity against both pathogens with inhibition zone and 15.10 mm (*E. coli*) and 18.75 mm (*S. aureus*) at the concentration 400 μg/disk, so it can be developed as a source of new drug.

4. Conclusion

Isolates KN-15-3 produces antibiotic that inhibited the MDR *S. aureus* and *E. coli.* Based on the morphological analysis and molecular analysis the fungus isolate KN-15-3 identified as *Aspergillus*

sydowii. The morphological and spicular characteristics indicated that the sponge host, KN-15 was Axinella sp.

5. Acknowledgement

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References

[1] Trianto A, Hermawan I, de Voogd N J, and Tanaka J 2011 Halioxepine, a new meroditerpene from an Indonesian sponge *Haliclona* sp. *Chemical & Pharmaceutical Bulletin*, 59(10), 1311–3.
[2] Li Z 2009 Advances in Marine Microbial Symbionts in the China Sea and Related Pharmaceutical Metabolites. *Mar. Drugs*, 7: 113-129.
[3] Sik W, Ki H, Young K, Am S, Soo Y, and Hee I 2006 Antifungal activity of synthetic peptide derived from halocidin, antimicrobial peptide from the tunicate, Halocynthia aurantium. *FEBS Letters*, 580: 1490–1496.
[4] Qaralleh H, Idid S, Saad S, Susanti D, Taher M, and Khleifat K 2010 Antifungal and Antibacterial Activities of Four Malaysian Sponge Species (Petrosiidae). *J. Med Mycology*, 20(4), 315–320.
[5] Yang L and Andersen R J 2002 Absolute Configuration of the Antiinflamatory Sponge Natural Product Contignasterol, 1924–1926.
[6] Fattorusso C, Persico M, Basilico N, Taramelli D, Fattorusso E, Scala F, andTaglialatela-Scafati O, 2011, Antimalarials based on the dioxane scaffold of plakortin. A concise synthesis and SAR studies. *Bioorganic & Medicinal Chemistry*, 19(1), 312–20.
[7] Trianto A, N J de Voogd, and J Tanaka 2014 Two new compounds from an Indonesian spons Dysidea sp., *J. Asian Nat. Prod. Res.*, 16(2):163-168.
[8] Mayer, A M S, Glaser K B, Cuevas C, Jacobs, R S, Kem W, Little R D, and Shuster D E, 2010 The odyssey of marine pharmaceuticals : a current pipeline perspective. *Trends Pharmacol Sci*., 31(6): 255-65
[9] Hadas E, Shpigel M, and Ilan M 2005 Sea ranching of the marine sponge Negombata magnifica (Demospongiae, Latrunculiidae) as a first step for latrunculin B mass production. *Aquaculture*, 244(1-4), 159–169.
[10] de Caralt S G, Agell M J and Uriz 2003 Long-term culture of sponge explants: conditions enhancing survival and growth, and assessment of bioactivity, Biomolecular Engineering 20. 339-347.
[11] Trianto A, Radisya N N, Wijayanti D P, Rifai A, Ismunarti D H, and Destio, 2013 Laju Pertumbuhan dan Kelulushidupan Transplan Spons Amphimedon sp., IJMS. Vol. 18(4):225-230
[12] Burgess J G, Boyd K G, Amstrong E, Jiang Z, Yan L, Berggren M, May U, Pisacane T, Granmo A, and Adams D R 2003 Development of a marine natural product-based antifouling paint. *Biofouling*., 19:197- 205.
[13] Hochmuth T and Piel J 2009 Polyketide synthases of bacterial symbionts in sponges--evolution-based applications in natural products research. *Phytochemistry*, 70(15-16), 1841–1849.
[14] Vasanthabharathi V and Jayalakshmi S 2011 Bioactive potential of symbiotic bacteria and fungi from marine sponges. *Afric J. Biotech*., 11(29): 7500-7511.
[15] Li Q and Wang G 2009 Diversity of fungal isolates from three Hawaiian marine sponges. *Microbiological Research*, 164(2): 233–41.
[16] Subramani R, Kumar R, Prasad P, Aalbersberg W, and Retheesh S T 2013 Cytotoxic and antibacterial substances against multi-drug resistant pathogens from marine sponge symbiont: Citrinin, a secondary metabolite of Penicillium sp. *Asian Pacific J Trop. Biomedicine*, 3(4), 291–296.
[17] Radjasa O K, Martens T, Grossart H P, Brinkhoff T, Sabdono A, and Simon M, 2007. Antagonistic Activity of a Marine Bacterium Pesueodaltromonas luteoviolacea TAB 4.2 Associated with Coral Acropora sp. *J. Bio. Sci*., 7(2): 239-246.
[18] Sponguide. Guide To Sponge Collection and Identification. *Order A Journal On The Theory Of Ordered Sets And Its Applications*, (Version).
[19] Almeida A P, Dethoup T, Singburaudom N, Lima R, Vasconcelos M H, Pinto M and Kijjoa A 2010 The in vitro anticancer activity of the crude extract of the sponge-associated fungus Eurotium cristatum and its secondary metabolites. J. Nat. Pharm. 1, 25–29.

[20] Safaeian S, Hosseini H, Abbas P A A, and Farmohamadi S 2009 Antimicrobial activity of marine sponge extracts of offshore zone from Nay Band Bay, Iran. *Journal de Mycologie Médicale / J. Med. Mycology*, 19(1), 11–16.
[21] Watanabe T 2002 Pictorial Atlas of Soil and Seed Fungi, Morphologies of Cultured Fungi and Key to Species. 2nd Ed. CRC Press. https://books.google.co.id/books/about/Pictorial_Atlas_of_Soil_and_Seed_Fungi.html?id 5ElPPhLN8JYC&redir_esc=y.
[22] Nursid M, Fajarningsih N D, and Chasanah E 2011 Screening of Antitumor Bioactivity of Fungi Associated with Macro Algae and Sponge from Indrayanti Beach , Jogjakarta, *Squalen Bulletin of Marine & Fisheries Postharvest & Biotech.*, *8*(2), 47–56.
[23] Alvarez B, Van Soest R W M, and Rutzler K 1998 A Revision of Axinellidae (Porifera: Demospongiae) of the Central West Atlantic Region. *Smithsonian Contributions to Zoo.*, 598(598): 1–47.
[24] Hamill P, Hudson D, Kao R Y, Chow P, Raj M, Xu H, Richer M J, and Jean F 2006 Development of a red-shifted fluorescence-based assay for SARS-coronavirus 3CL protease: identification of a novel class of anti-SARS agents from the tropical marine sponge Axinella corrugata. *Biol Chem.*387(8): 1063-74.
[25] Dai J, Fishback J A, Zhou Y D, and Nagle D G 2006 Sodwanone and yardenone triterpenes from a South African species of the marine sponge Axinella inhibit hypoxia-inducible factor-1 (HIF-1) activation in both breast and prostate tumor cells. *J. Nat Prod*, 69(12): 1715-20.
[26] Aiello A, Fattorusso E, Giordano A, Menna M, Müller W E, Perovic-Ottstadt S, and Schroder H C 2007 Damipipecolin and damituricin, novel bioactive bromopyrrole alkaloids from the Mediterranean sponge Axinella damicornis. *Bioorg. Med. Chem*, 15: 5877–5887.
[27] Sakai R, Matsubara H, Shimamoto K, Jimbo M, Kamiya H, and Namikoshi M 2003 Isolations of N-methyl-d-aspartic acid-type glutamate receptor ligands from Micronesian sponges. *J. Nat. Prod.*, 66:784–787.
[28] Yalçın F N, Ohno T, Ersöz T, Saracoğlu İ, Çalış İ, Inoue M, and Ogihara Y 2002 Cytotoxic and Antioxidant Activities of the Eastern Mediterranean Sponges Ircinia and Axinella. Hacettepe University, *J Faculty of Pharmacy*, 22: 19.
[29] Moťková P and Vytřasová J 2011 Comparison of Methods for Isolating Fungal DNA. *Czech J. Food Sci*, 29: S76–S85.
[30] Bennett J W and Papa K E 1988. The aflatoxigenic Aspergillus spp. In: Sidhu G.S. (ed.): Advances in Plant Pathology, No. 6. Academic Press, London: 263–280.
[31] Mayer Z , Bagnara A, Färber P, and Geisen R , 2003 Quantification of the copy number of nor-1, a gene of the aflatoxin biosynthetic pathway by real-time PCR, and its correlation to the cfu of Aspergillus flavus in fous. *Int. J. Food Microb*, 82: 143– 151.
[32] Goto T, Wicklow D T, and Ito Y 1996 Aflatoxin and cyclopiazonic acid production by a sclerotium-producing Aspergillus tamarii strain. *Applied and Environmental Microbiol*, 62: 4036–4038.
[33] Villa P and Markaki P 2009 Aflatoxin B1 and ochratoxin A in breakfast cereals from Athens market: Occurrence and risk assessment. *Food Control*, 20: 455–461.
[34] Yang Z, Shim W B, Kim J H, Park S-J, Kang S, Nam B, and Chung D 2004 Detection of aflatoxin-producing molds in Korean fermented foods and grains by multiplex PCR. *J. Food Protection*, 67: 2622–2626.
[35] Malmstrøm J, Polson S C, Polson S W, Smith G W, and Frisvad J C 2001 Study of secondary metabolites associated with virulent and non-virulent strains of Aspergillus sydowii: Sea fan pathogen. Pages 48-52 in Proceedings of the Symposium on the Natural History of the Bahamas. Gerace Research Center, San Salvador, Bahamas.
[36] Bugni T S and Ireland C M 2004 Marine-derived fungi: a chemically and biologically diverse group of microorganisms. *Nat. Prod.*, 21: 143–163.
[37] Swathi J Narendra K Sowjanya K M Satya A K 2013 Marine fungal metabolites as a rich source of bioactive compounds. *Nat. Prod*, 21: 143–163.
[38] Choudhary M I Musharraf S G Mukhmoor T Shaheen F Ali S Rahman A 2004 Isolation of Bioactive Compounds from Aspergillus terreus. *Z. Naturforsch.* 59b: 324 – 328.
[39] Blunt J W Copp B R Keyzers R A Munro M H G Prinsep M R 2015 Marine natural products. *Nat. Prod. Rep.*, 32 : 116-211.
[40] El-hady F K A Abdel-aziz M S Shaker KH. El-shahid ZA 2014 Tyrosinase, Acetylcholinesterase Inhibitory Potential, Antioxidant and Antimicrobial Activities of Sponge Derived Fungi with Correlation to Their GC/MS Analysis. *Int. J. Pharm. Sci. Rev. Res.* 26(58): 338–45.