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Comparative Study of the Secondary Metabolites of Sponge-derived *Aspergillus flavus*

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Abstract

The fungal sources of novel metabolites have broadened from saprophytic terrestrial strains to marine habitats and living plants with their endophytes. Specifically, metabolites isolated from species of genus *Aspergillus* have continually attracted the interest of pharmacologists due to their broad array of biological activities and their structural diversity.

Bioassay guided fractionation of the extract of saline culture of marine-derived *Aspergillus flavus* led to the isolation of eight compounds; kojic acid (1), aflatoxin B1 (2), maculosin1 (3), hexahydro-3-(4-hydroxybenzyl)pyrrolo[1,2-a]pyrazine-1,4-dione (4), cyclopiazonic acid (5), cyclopiazonic acid imine (6), aspergillic acid (7) and hydroxyaspergillic acid (8). Structure elucidation of the compounds was based on dereplication using NMR and MS data. A cultivation-based approach was employed to compare the secondary metabolites diversity associated with *A. flavus* in eight sea water culture media. The type of medium exhibited a significant difference in the yield and the chemistry of compounds responsible for biological activities of the corresponding extract.

1. Introduction

The quest to exploit factors leading to the production of diverse molecular structures from cultured microorganisms represents a continuing challenge for natural products research (Firn and Jones, 2003) Filter-feeding marine invertebrates, such as sponges, have been shown to host a variety of microorganisms that do not merely reflect the microbial communities present in the surrounding seawater but appear to constitute a more specialized association between sponge hosts and microbial associates (Friedrich et al, 1999). An early, conservative estimate based upon thousands of assayed sponge species suggested that as many as 11% produce cytotoxic compounds (Garson, 1994). This percentage is high compared to other organisms and possibly may result from the intimate association between sponges and diverse microbial communities (Sipkema, 2009). Sponge-derived fungal cultures have been shown to be interesting sources of bioactive secondary metabolites previously unknown from terrestrial strains of the same species. The genus *Aspergillus* has been repeatedly been isolated from marine sources such as sponges or algae (Hiort et al, 2004). Although their ecological roles in the sea are not well defined, the genus *Aspergillus* has been identified as the causative agents for mass mortalities in marine invertebrates (Smith, 1996) providing evidence that these fungi can produce significant ecological effects (Garo, 2003). The probability of finding useful metabolites from marine organisms is obviously dependent on the number and quantity of samples screened. Therefore working on symbionts instead of marine macroorganisms has further expanded the diversity of structures that can be obtained without spending great efforts to collect vast amounts of the marine macro creatures. In addition, the increased awareness of the potential of marine

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biological resources has highlighted the need for nations to become aware of biological diversity and the importance of conservation of these resources. In our study we report the isolation of fungi associated with the sponge *Haliclona (Gellius) bubastens*. Several marine derived fungi were identified by the use of DNA sequencing combined with World Wide Web (WWW) searchable databases.

The sponge derived *A. flavus* was chosen for chemical and biological investigation of its secondary metabolites in saline cultures. In addition, the effect of changing the carbon source in culture media of the studied fungus on metabolites production and diversity was also studied. Eight saline culture media were prepared; czapek's dox yeast broth (CZYB), four modified czapek's dox yeast (M.CZYB), Sabouraud dextrose broth (SDB), malt broth (MB) and yeast peptone dextrose broth (YPDB) in order to find the optimal culture composition for *A. flavus* to produce its biologically active metabolites in respect to concentration and diversity.

2. Materials and methods

2.1. General

NMR experiments were carried out at 25 °C using either a 400 MHz or 500 MHz Varian INOVA-NMR spectrometer with standard pulse sequences. UV spectra were recorded on an *hp*-8552A diode array spectrophotometer. Liquid chromatography-mass spectrometry (LC/MS) with electrospray ionization mass spectrometry (ESI-MS) was run at the University of Utah Mass Spectrometry and Proteomics Core Facility. DNA sequencing was done using high throughput capillary electrophoresis instrument (ABI 3730XL) at the University of Utah DNA sequencing and genomics core facility. HPLC-Hitachi Ez lachrome equipped with C_{18} columns (10 x 250 mm and 4.6 x 150 mm) and photodiode array detector was used for compounds isolation and analysis.

2.1. Sponge material

The host sponge was collected in the Red Sea and was identified as *Haliclona (Gellius) bubastens* (Row, 1911).

2.2. Isolation of sponge associated fungi

In order to ensure fungal isolates to be sponge-associated or symbiotic, surface sterilization of the collected sponge had to be made. A piece of sponge tissues (1 g) was washed with 5% sodium hypochlorite followed by 70% ethanol and finally with sterile artificial sea water (ASW). Small pieces of pre-washed sponge tissues were inoculated onto different agar media; malt Agar (MA, Difco), Sabouraud dextrose Agar (SDA, Difco), Czapek's Dox yeast Agar (CZYA (g/L each) sucrose (30), yeast (5), $NaNo_3$ (3), K_2HPo_4 (1), KCl (0.5), $MgSo_4.7H_2O$ (0.5), $FeSo_4.7H_2O$ (0.01)), yeast peptone dextrose Agar (YPDA (g/L each) yeast (10), peptone (20), dextrose (20)).

2.3. Identification of fungal isolates

Fungal DNA was prepared using DNeasy Plant Mini Kit (Qiagen). The prepared DNA was amplified with polymerase chain reactions (PCR) followed by agarose gel electrophoresis. DNA was then retrieved from the agarose gel with QIAquick gel extraction kit (Qiagen). DNA sequencing combined with WWW searchable databases was used for fungi identification.

2.4. Extraction and isolation

A. flavus was chosen for large scale culture. 20 L of CZYB in ASW were prepared and inoculated with the studied fungus. The prepared cultures were kept at 27 ºC with shaking at 120 rpm for 14 days. Extraction of the filtered broth with EtOAc gave 3.1 g crude extract which was further partitioned between 10% aqueous MeOH and hexane. The hexane fraction was separated and evaporated to give 1 mL of red oil (586.6 mg). The polarity of the aqueous portion was raised to 50 % aqueous MeOH and partitioned with dichloromethane (DCM). DCM fraction was separated and dried under vacuum to give 794 mg.

DCM extract (794 mg) was applied to a column of sephadex-LH_{20} and eluted with MeOH:DCM (1:1) to give three different fractions (F_1-F_3). F_2 showed the most intense biological activities against the tested bacteria and fungi. F_2 was applied to the top of several silica gel columns (A-C) and step gradient elution with hexane, chloroform and EtOAc was used. Two fractions from columns A and C were crystallized from saturated chloroform solution to give **1** (2.5 mg) and **2** (1.5 mg), respectively. Bioassay guided fractionation led to the identification of two major fractions from column C. FC-1 (12 mg) was cytotoxic to brine shrimps in a concentration of 150 µg. RP-HPLC of FC-1 resulted in the separation of **3** (1.5 mg), **4** (1mg), **5** (5 mg) and **6** (1mg). FC-2 (6 mg) showed antimicrobial properties. Further purification of FC-2 with RP-HPLC gave rise to the separation of **7** (800 µg) and **8** (1.2 mg). The structures of the isolated compounds were elucidated using 1D and 2D NMR spectroscopic data together with ESI-TOF-MS

data (**Figure 1**).

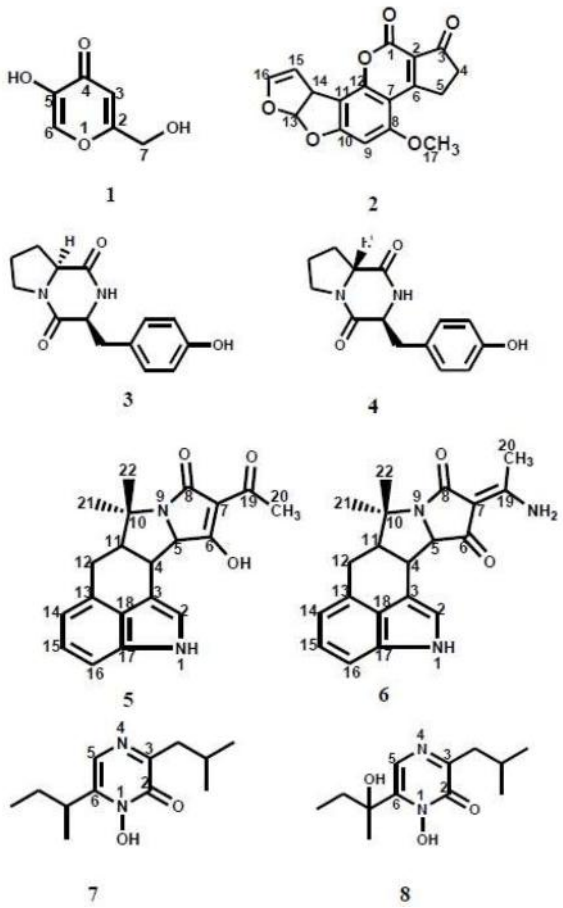

Figure 1: Structures of isolated compounds (1-8)

2.5. Preparation and extraction of eight culture media for comparative study

A. flavus was left to grow until it began to sporulate, and yellowish green color can be seen covering most of the petri dish. After 1 week, 200 µL sterile CZYB were poured over the plate and mixed well by repeated pipetting. 20 µL of the produced spore suspension was inoculated into eight different culture media prepared in 50% ASW (500 mL each, (g/L)); CZYB **(**sucrose (30), yeast (5), $NaNO_3$ (3), K_2HPO_4 (1), KCl (0.5), $MgSo_4.7H_2O$ (0.5), $FeSo_4.7H_2O$ (0.01)**)**, M.CZYB$_1$ **(**glucose (30)**)**, M.CZYB$_2$ **(**fructose (30)**)**, M.CZYB$_3$ **(**lactose (30)**)**, M.CZYB$_4$ **(**starch (30)**)**, MB **(**Difco**)**, SDB **(**Difco**)** and YPDB **(**yeast (10), peptone (20), dextrose (20)**)**. M.CZYB contains all of CZYB components except that the carbon (carbohydrate) source was changed using mono-, di- and polysaccharides. The eight prepared cultures were kept at 27 ºC with shaking at 120 rpm. After two weeks of shaking and incubation, each liquid culture was filtered using clean gauze to separate the mycelia from the broth. The mycelia were soaked in 200 mL MeOH for 48 h while the broth was extracted with EtOAc (3 x 500 mL). These extracts were evaporated to dryness under vacuum and weighed (**Table 1**).

Table 1: The yield of crude extracts from the eight studied saline cultures (500 mL each)

Culture media	MeOH extract	EtOAc extract
CZYB	273	103
M.CZYB1	208	50
M.CZYB2	373	72
M.CZYB3	418	60
M.CZYB4	-	47
MB	435	113
SDB	347	126
YPDB	139	354

Numbers show the weight in mg

2.6. Preparation of standard mixture and HPLC analysis

A standard mixture of the compounds **1**, **2**, **3**, **5** and **8** was prepared (1 µg/µL each, in 100 µL MeOH-H_2O) and analyzed by RP-HPLC using a C_{18} column (4.6 mm x 150 mm). Compounds were eluted with a linear gradient from 95% solvent A (0.05% trifluoroacetic acid in water) to 95% solvent B (0.05% trifluoroacetic acid in acetonitrile) over 70 min at 1 mL/min (**Figure 2**). Each compound was applied separately to C_{18} HPLC analytical column using the proposed method and purity of each compound was checked and found to be more than 98%. EtOAc extract of each of the eight cultures (100 µg/µL) was analyzed using the previously described method. Quantitative

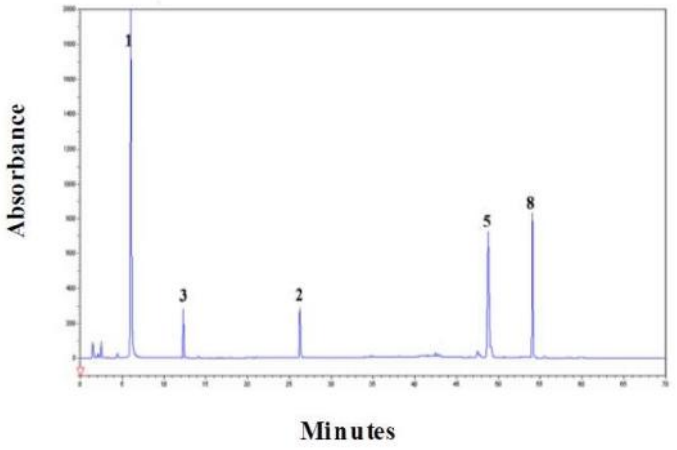

Figure 2: HPLC chromatogram of the prepared standard mixture of some of the isolated A. flavus metabolites showing peaks of: 1 at 6.0 min, 3 at 12.3 min, 2 at 26.2 min, 5 at 48.7 min and 8 at 54.1 min

Table 2: Results of RP-HPLC of EtOAc extract of eight cultures (w/w) (concentrations are in micrograms (µg) present in 1 mg of extract according to peak area measurement)

	1	3	2	5	8
CZYB	0.22	4.18	29.80		6.47
M.CZYB1		16.08	54.81	3.30	7.64
M.CZYB2			38.89	9.36*	7.34
M.CZYB3	2.69			5.10	8.52*
M.CZYB4	3.34		25.70	0.68	6.52
MB	7.09*		86.45*		6.51
SDB		167.12*	43.65	6.58	6.37
YPDB					7.90

determination of each of the compounds **1**, **2**, **3**, **5** and **8** in extracts were done using peak area measurement (**Table 2, Figure 3**).

2.7. Biological assays

The bactericidal and fungicidal activitities of EtOAc and MeOH extracts were tested against *E.coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Candida albicans*, *Saccharomyces pombe* and *Saccharomyces cervisiae* using agar disc diffusion assay (Anderson, 1978). The cytotoxic properties of these extracts were examined using brine shrimp lethality assay (Kapadia et al, 2002; Pimenta et al, 2003). The lethal dose required to kill 50 % of brine shrimps population (LD_{50}) was calculated.

Spectroscopic data of the compounds

Kojic acid (1)

UV/Vis λ_{max} (H_2O) nm (log ε): 268 (0.9)

^{1}H NMR (400 MHz, CD_3OD): 4.40 (2H, s, Methylene), 5.49 (H, s, OH), 6.48 (1H, s, H-3), 7.95 (1H, s, H-7).

MS (EI, 70 eV): m/z = 142.9 [M + H^+] for ($C_6H_6O_4$), 164.9 [M+Na]$^+$.

Aflatoxin B1 (2)

UV/Vis λ_{max} (EtOH) nm (log ε): 223 (1.4), 265 (1.1), and 362 (1.3).

^{1}H NMR (400 MHz, $CDCl_3$): 2.62 (2H, d, H-4), 3.38 (2H, d, H-5), 3.95 (3H, s, H-17), 4.78 (1H, dd, *J* = 7.0, 3.0 Hz, H-14), 5.45 (1H, t, *J* = 3.0, 3.0 Hz, H-15),

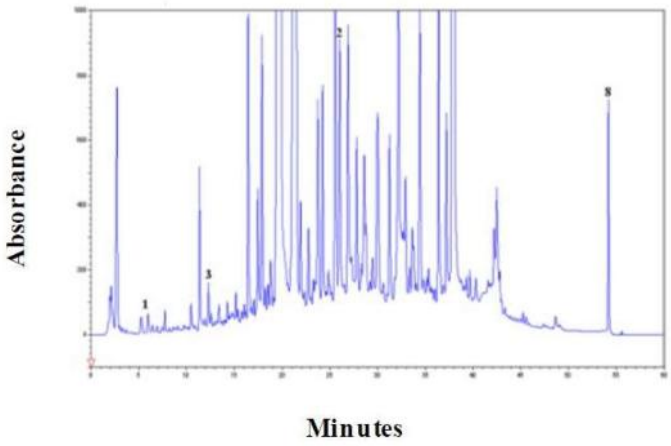

Figure 3: HPLC chromatogram of EtOAc extract of *A. flavus* cultured in saline CZYB showing peaks of: 1 at 5.9 min, 3 at 12.3 min, 2 at 26.1 min and 8 at 54.1 min

6.42 (1H, s, H-9), 6.51 (1H, t, H-16), 6.85 (1H, d, *J* = 7.0 Hz, H-13).

MS (EI, 70 eV): m/z = 313.3[M+H]$^+$ for ($C_{17}H_{12}O_6$).

Maculosin 1 (3) and Hexahydro-3-(4-hydroxybenzyl)pyrrolo[1,2-a]pyrazine-1,4-dione (4)

UV/Vis λ_{max} (H_2O) nm (log ε): 278 (1.2).

^{1}H NMR (500 MHz, D_2O): Pyrazine-1,4-dione, 3.95 (1H, dd, H-6), 4.42 (1H, dd, H-3), 3-(4-hydroxybenzyl), 2.95 (1H, dd, methylene), 3.09 (H1, dd, methylene), 5.38 (1H, s, OH), 6.70 (2H, d, H3 and H5), 6.91 (2H, d, H2 and H6), Pyrrole, 0.60 (1H, m, H-4), 1.92 (1H, m, H-4), 1.7 (2H, m, H-3), 3.2 (1H, ddd, H-2), 3.42 (1H, ddd, H-2).

^{13}C NMR (125 MHz, D_2O): 21.2 (CH_2), 28.3 (CH_2), 38.4 (CH_2), 45.0 (CH_2), 57 (CH), 59.3 (CH_2), 115.5 (CH), 132.3 (CH).

MS (EI, 70 eV): m/z = [M+H]$^+$ 261.096 for ($C_{14}H_{16}O_3N_2$).

Cyclopiazonic acid (5) and **cyclopiazonic acid imine (6)**

UV/Vis λ_{max} (MeOH) nm (log ε): 225 (0.9) and 253 (1.3).

^{1}H NMR (500 MHz, $CDCl_3$): 1.66 (3H, s, H-21) and (3H, s, H-22), 2.45 (3H, s, H-20), 2.62 (1H, dd, H-11), 3.04 (2H, d, H-12), 3.64 (1H, dd, H-4), 4.05 (1H, d, H-5), 6.90 (1H, d, H-14), 7.16 (1H, t, H-15), 7.18 (1H, d, H-2), 7.22 (1H, d, H-16).

^{13}C NMR (125 MHz, $CDCl_3$): 19.2 (CH_3), 24.3 (CH_3),

25.5 (CH_3), 26.0 (CH_2), 35.5 (CH), 52.5 (CH), 71.9 (CH), 108.4 (CH), 115.9 (CH), 120.4 (CH), 122.8 (CH).

MS (ESI): m/z = (5) $[M+H]^+$ 337 for ($C_{20}H_{20}O_3N_2$) and (6) $[M+H]^+$ 336 for ($C_{20}H_{21}O_2N_3$).

Aspergillic acid (7) and hydroxyaspergillic acid (8)

UV/Vis λ_{max} (H_2O) nm (log ε): 278 (1.2).

^{1}H NMR (500 MHz, CD_3OD): Pyrazinone, 4.56 (1H, s, N-OH), 7.9 (1H, s, H5), 3-(2-methylpropyl), 0.83 (6H, d, methyl), 2.05 (1H, ddsept, methine), 2.62 (2H, dd, methylene), 6-(1-methylpropyl), 0.68 (3H, t, methyl) and 1.68 (3H, s, methyl), 0.95 (1H, methine, **7** only), 1.92 (1H, q, methylene), 2.38 (1H, q, methylene).

^{13}C NMR (125 MHz, CD_3OD): 9.4 (CH_3), 23.3 (CH_3), 25.6 (CH_3), 29.1 (CH), 33.4 (CH_2), 42.2 (CH_2), 125.7 (CH), 146.2 (C6), 150 (C2 and C3).

MS (ESI): m/z = (**7**) [M+H]+ 225.1 for ($C_{12}H_{20}O_2N_2$) and (**8**) [M+H]+ 241 for ($C_{12}H_{20}O_3N_2$).

3. Results and Discusion

Eight compounds (**Figure 1**) including kojic acid (**1**) (Basappa et al,1970), aflatoxin B1 (**2**) (Basappa et al,1970; Van,1970), maculosin1(**3**) (Stierle et al, 1988; Rudi and Kashman,1994), hexahydro-3-(4-hydroxybenzyl) pyrrolo[1,2-a]pyrazine-1,4-dione (**4**) (De Rosa et al, 2003), cyclopiazonic acid (**5**) (Holzapfel and Hutchison, 1970), cyclopiazonic acid imine (**6**) (Holzapfel and Hutchison, 1970), aspergillic acid (**7**) (Dutcher, 1947; Wilson, 1966; Perry et al) and hydroxyaspergillic acid (**8**) (Wilson, 1966; Perry et al, 1984; Dutcher, 1958) were isolated and purified from the ethyl acetate (EtOAc) extract of CZYB saline culture. These compounds have reported biological activities; cytotoxic, antibacterial, antifungal and pesticide properties (Basappa et al,1970; Van,1970; Stierle et al, 1988; Rudi and Kashman,1994; De Rosa et al, 2003; Holzapfel and Hutchison, 1970; Dutcher, 1947; Wilson, 1966; Perry et al; Dutcher, 1958; Bobylev et al,1996). Extraction of the eight saline culture media of *A. flavus* with organic solvents and bioassay of the obtained crude extracts showed a significant effect of the nutritional environment on the produced secondary metabolites (**Table 1-2, Figures 3-5**). The results of quantitative determination of the compounds **1**, **2**, **3**, **5** and **8** in EtOAc extracts of the eight prepared cultures by HPLC revealed significant variations of the concentration of these compounds between the studied extracts (**Table 2, Figures 2-3**). Analysis of HPLC data using the proposed method with peak area measurement revealed that *A. flavus* synthesized higher concentration of compound **3** in SDB than it did with any other substrate tested. Although compound **3** is produced only in three of the eight studied culture media, its concentration differs significantly between their extracts. It was found in SDB culture extract in forty and ten folds its concentration in CZYB and M.CZYB$_1$, respectively. The concentration of compound **1** in EtOAc extract of MB was found to be more than two and thirty fold its concentration in M.CZYB$_3$, M.CZYB$_4$ and CZYB, respectively (**Table 2**). Compounds **1** and **2** were synthesized in the highest concentrations in MB**.** The production of compound **2** was three fold more in MB than in CZYB and M.CZYB$_4$ while it is not produced in cultures of M.CZYB$_3$ and YPDB (**Table 2**). Compound **5** is present in cultures of M.CZYB$_2$ with the highest percentage while it is not produced in CZYB, MB and YPDB culture media. Compound **5** was found at the lowest concentration in M.CZYB$_4$ and at different proportions in each of M.CZYB$_1$, M.CZYB$_3$ and SDB (**Table 2**). Compound **8** is produced in all of the eight studied cultures with the highest percentage in M.CZYB$_3$. No significant variation in the production of compound **8** in all of the studied culture media. The biological activities of the methanolic (MeOH) and EtOAc extracts of the studied cultures were tested for antimicrobial and cytotoxic properties. Analysis of results of the antimicrobial assays revealed that some extracts of the studied cultures showed antibacterial and antifungal activities as potent as or even better than chloramphenicol and nystatin, respectively, at the same tested concentration. EtOAc extract of SDB showed the highest activity against *B. subtilis* and *S. aureus* which is higher than the activity of chloramphenicol at the same tested concentration (**Figure 4**). 400 µg of EtOAc extracts of CZYB and M.CZYB$_3$ showed the same fungicidal activity against *C. albicans* while they were one and half fold more potent than nystatin against *S. pombe*. Moreover, EtOAc extract of SDB gave the same inhibition zone against both *C. albicans* and *S. cervisiae* as nystatin at the same tested concentration (**Figure 4**). Close analysis of the results of antimicrobial assays of extracts of all studied cultures revealed that sponge-derived *A. flavus* favored the synthesis of both antibacterial and antifungal metabolites in saline SDB. Cytotoxic activities of the prepared extracts were tested with the rapid and efficient brine shrimp lethality assay. Results showed a significant difference of their LD_{50}. The EtOAc extract of M.CZYB$_1$ showed the lowest LD_{50} (as low as 2 µg), while more than 1 mg of its MeOH extract

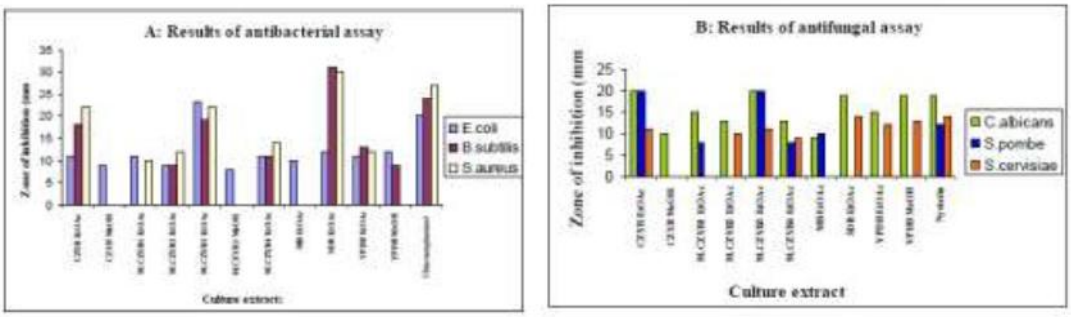

Figure 4: Results of antimicrobial assays of extracts of the eight studied cultures (400 µg each); A: antibacterial assay using chloramphenicol as a reference standard, B: antifungal assay using nystatin as a reference standard.

was required to kill 50% of brine shrimps population. 20 µg was calculated for LD_{50} of the MeOH extract of YPDB, while no cytotoxic activity was noticed for the MeOH extract of $M.CZYB_2$ (**Figure 5**).

A good explanation of these results is that the secondary metabolites responsible for the cytotoxic properties are released extensively in the culture media of $M.CZYB_1$ and SDB. This situation is clearly opposite in case of YPDB, where the cytotoxic compounds are present extensively inside the mycelia of the studied fungus (**Table 2, Figure 5**).

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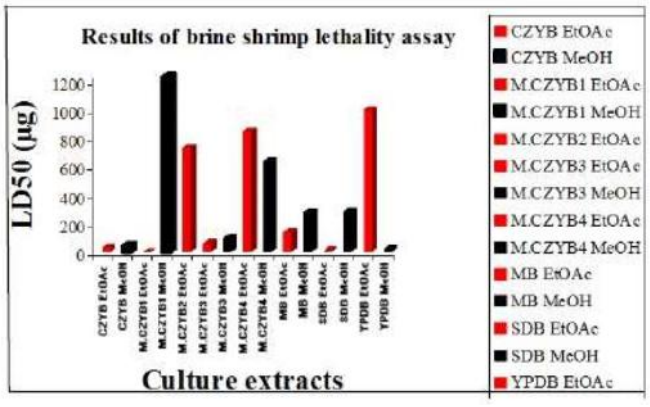

Figure 5: Results of brine shrimp lethality assay of extracts of the eight studied cultures

4. Conclusions

In conclusion, marine fungi are considered an environmentally friendly source of pharmacologically-active natural products that allow the conservation of marine biological resources. Therefore, chemical and biological investigation of the secondary metabolites of the sponge-associated *A. flavus* in eight culture media was extensively studied to find the optimal culture composition for their production. Large scale culture of *A. flavus* led to isolation of eight compounds. The isolated compounds represent a wide diversity in their chemical structures and sizes. Their complexity varies from Kojic acid, γ-pyrone derivative, to the tetramic acid, cyclopiazonic acid. Analysis of HPLC data and bioassays results of extracts of the eight studied culture media showed a significant effect of the nutritional environment on the production of secondary metabolites in respect to concentration and diversity.

5. Acknowledgments

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6. Conflict of interest

The authors report no declaration of conflict of interest.

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