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Isolating and Screening Mangrove Microalgae for Anticancer Activity

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ABSTRACT

Microalgae are valuable source of many unique biologically active compounds including anticancer compounds. In this study, sixteen microalgal strains were successfully isolated from mangrove in Xuanthuy National Park, Namdinh, Vietnam. Relative identification for each train was obtained based on morphological properties and 18 S rDNA sequence analysis. Culture extracts of these strains were tested against KB (human epidermal carcinoma) cell line. *Ankistrodesmus gracilis* VACC-010 and *Amphiprora alata* VACC-007 showed strongest inhibition with their IC_{50} values of 26.50 and 29.82 μg mL^{-1}, respectively. *Ankistrodesmus gracilis* VACC-010 was also significantly effective against HepG2 (hepatocellular carcinoma) cell line (IC_{50} values of 9.64 μg mL^{-1}), suggesting a potential source of anticancer compounds. To our knowledge, this is the first report on the anticancer activity of this strain as well as the first screening of mangrove microalgae for biologically active compounds, providing a new lead to the characterization and development of promising anticancer drugs.

Key words: *Amphiprora alata*, *Ankistrodesmus gracilis*, anticancer activity, mangrove, microalgae, screening

INTRODUCTION

Microalgae are a highly diversified group of microorganisms, which are mostly unicellular, colorful, photoautotrophic and constitute major oceanic as well as freshwater primary producers (Olaizola, 2003). They have been utilized by man for hundreds of years in various fields ranging from human and animal nutrition, cosmetics to therapeutic purposes. They do possess high-value compounds such as carotenoids, polyunsaturated fatty acids, vitamins and many biologically active compounds (Spolaore *et al*., 2005). Previous investigations of microalgae have shown that they are promising sources for a wide range of novel biologically active molecules with antibacterial, antiviral, antifungal and anticancer activities (Ghasemi *et al*., 2007). As microalgae are assumed to be easier to culture commercially than macroalgae, they have the potential to produce those compounds which are difficult to synthesize (Borowitzka, 1995).

Approximately 60% of new drugs for cancer treatment are derived from natural sources. Many of them have been found in cyanobacteria, which used to be considered as blue-green algae, such as calothrixin A and B (*Calothrix* spp.), apratoxin A, curacin-A (*Lyngbya majuscula*), largazole (*Symploca* sp.), borophycin (*Nostoc linckia*, *N. spongiaeforme* var. *tenue*) (Boopathy and Kathiresan, 2010; Baharum *et al*., 2010; Vignesh *et al*., 2011). Many compounds are active in either killing the cancer cells by blocking cancer cell growth and inducing apoptosis or affecting the cell signaling through the activation of protein kinase family members (Borowitzka, 1995;

Khorshid *et al.*, 2011). They have unprecedented structures thus can be the potential for the development of new classes of drug agents. However, other microalgal groups have gained increasing interest and become promising sources for exploration. Mechanism based screening for potential anticancer activity including protein kinase C, protein tyrosine kinase and inosine monophosphate dehydrogenase assays has found a range of potential candidates from various microalgae such as Rhodophyta, Chlorophyta, Phaeophyta, Chrysophyta and Cryptophyta (Gerwick *et al.*, 1994). Noda *et al.* (1996) purified and identified a glycoprotein from the culture media of *Chlorella vulgaris* with a β-1, 6-D-galactopyranose backbone and 15 amino acid sequence as DVGEAFPTVVDALVA at the NH_2-terminus, which was considered necessary for the antitumor activity. The vitamin extracts of 7 chlorophyte strains were proved to be potential chemopreventive agents by inducing the activity of detoxifying enzyme glutathione-S-transferase in many tissues of tumorous mice (El-Baz *et al.*, 2002). Recent survey of nearly two hundred microalgal strains, resulted in ten chlorophytes from three genera *Desmococcus*, *Chlorella* and *Scenedesmus* with high antimicrobial activity and effective against some tumour cell lines such as MCF7 (human breast adenocarcinoma), CEM (human lymphoblastoid leukaemia) and G361 (human malignant melanoma) (Ordog *et al.*, 2004).

Mangroves are one of the most productive ecosystems, a rich source in biodiversity including phytoplankton and have long been used in traditional medicine for many diseases (Datta *et al.*, 2011). Many mangrove plants were reported to be good sources of anticancer drugs (Boopathy and Kathiresan, 2010). In an attempt to search for biologically active compounds as potential anticancer drug agents in various microalgal species from mangrove, this research focused on the isolation of various microalgal strains from the mangrove of Xuanthuy National Park, Namdinh, Vietnam, their identification and laboratory culture and crude extraction thereafter for assays against several cancer cell lines.

MATERIALS AND METHODS

Selection, isolation and identification of microalgal strains: Samples were collected from different sites of mangrove in Xuanthuy National Park, Namdinh, Vietnam from March to October, 2010 and cultured in 10 mL jars of f/2 medium. Microalgal strains were isolated to a unialgal state using micropipettes and agar plates by the procedure according to Shirai *et al.* (1989). Each strain was taken picture under 400-fold OLYMPUS CX41 microscopy. Biomass culture for anticancer activity assays was carried out in 500 mL conical flasks and then in 4 L flat-bottom round flasks at room temperature with illumination by neon light (Philips daylight tubes) of 3000-4000 lux on 10: 14 h light: dark cycles. Chlorophytes were grown in BBM, C, BG11 media and the other strains in f/2, ASW, ESM media (Kasai *et al.*, 2009).

Total DNA was extracted and PCR amplification was performed according to the method described by Fawley and Fawley (2004) using following primers:

- **Forward primers 2F:** (2-21) 5'-ATCTGGTTGATCCTGCCAGT-3' or 1315 F: 5'-CGATAAGGAACGAGACCTT-3'
- **Reverse primer 1794R:** (1794-1775) 5'-GATCCTTCCGCAGGTTCACC-3'

PCR products were directly sequenced in an ABM Prism 3100-Avant Sequencer. The obtained sequences were analyzed using BLASTn tool to get the relative identification of each algal species.

Preparation of microalgal extracts: Algal samples were harvested in early stationery phase (around 11-12th day of culture) by continuous centrifugation at 10000 rpm at 4°C in 15 min and

extracted thereafter with 10 mL of methanol/chloroform (1:1, v/v). The extracts were concentrated under vacuum to give residues and then stored at -20°C until analyzed.

Anticancer assay: Microalgal extracts were tested against KB (human epidermic carcinoma) and then against HepG2 (hepatocellular carcinoma), SK-LU-1 (human lung carcinoma) and MCF-7 (human breast carcinoma) cell lines from ATCC (American Type Culture Collection) using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Scudiero *et al*., 1988). Cell lines were cultured in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS) in standard condition, steriled with 5% CO_2 at 37°C, 98% humidity and harvested at the log phase for assays. Two hundred microliter volumes of cells at the concentration of 3×10^4 cells mL^{-1} were inoculated into a 96-well plate in RPMI 1640 medium. Microalgal extracts were applied at final concentrations of 128, 32, 8, 2 and 0.5 μg mL^{-1} and cultures were incubated for 3 days at 37°C with 5% CO_2. Then 50 μL of MTT prepared at the concentration of 1 mg mL^{-1} in FBS was added to microculture wells. After 4 hours incubation, 250 μL supernatant were removed from each well and 100 μL of DMSO was added and mixed thoroughly. Absorbance was measured at 540 nm in a Genios TECAN spectrophotometer. IC_{50} value (extract concentrations resulting in a 50% inhibition of growth) was calculated based on the percentage of growth:

$$\text{Inhibition (\%)} = \frac{OD_{control} - OD_{sample}}{OD_{control}} \times 100$$

RESULTS AND DISCUSSION

Isolation and identification of microalgal strains: Fifteen microalgal strains (Fig. 1) belonging to 11 genera including 6 chlorophytes, 8 diatoms and 1 eustigmatophyte were selected

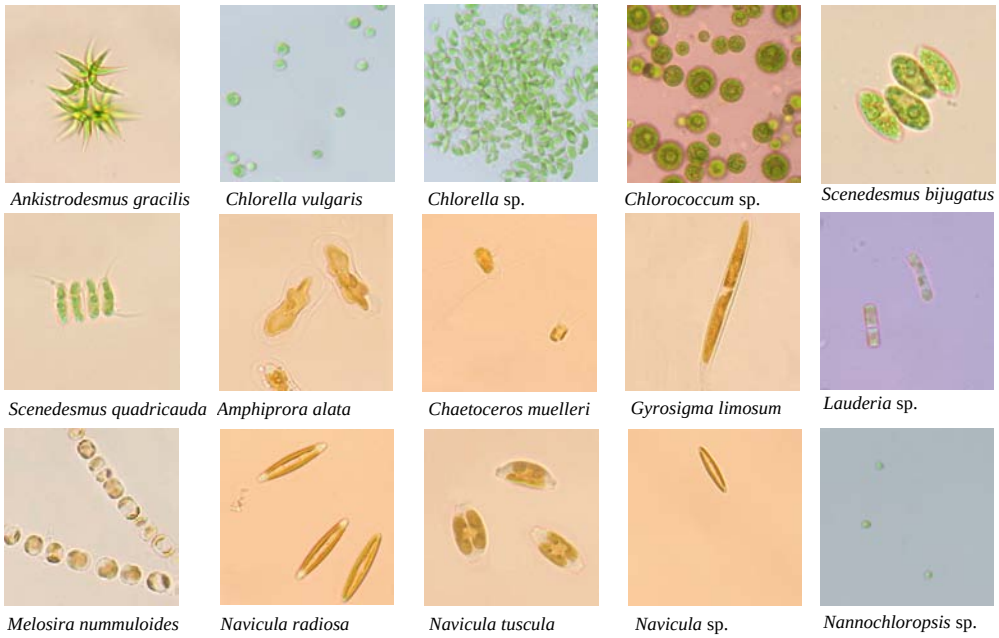

Fig. 1: Microscopic morphology of microalgal strains isolated from mangrove in Xuanthuy National Park

and isolated to a unialgal state according to standard literature procedures based on morphological properties (Shirora, 1966). Sequence analysis and alignment with sequences on NCBI database gave the positive identity for almost all strains. Data were shown in Table 1 together with short description of their morphology.

Isolated microalgal strains were cultured in several media: BBM, C and BG11 for chlorophytes and f/2, ESM and ASW for the other. The growth of all strains was promoted well in these media,

Table 1: Characteristics of microalgal strains isolated from Xuanthuy National Park

Strains (VACC)	Sequence length (bp)	Matched species	Morphology
010	679	*Ankistrodesmus gracilis*	Cells have crescent shape or sickle shape and are longer than broad and strongly curved or twisted. They can form non-mucilaginous colonies from clusters of 4, 8, or 16 cells which are arranged with their convex sides facing each other.
012	692	*Chlorella vulgaris*	This species usually lives as single cells with spherical shape and without flagella.
013	-	*Chlorella* sp.	Cells are symmetrical with chloroplast initially saucer and becoming more lobed with age. Pyrenoid is associated with numerous starch grains
018	-	*Chlorococcum* sp.	Cells are circular or spherical or elliptical. The cells may be solitary or in irregular clumps. Each cell has a single cup-shaped, parietal chloroplast with a single pyrenoid.
014	706	*Scenedesmus bijugatus*	Cells are oval or elipoval, big, laterally adjoin and arrange in linear series. They usually form colonies of 4-8 cells.
015	649	*Scenedesmus quadricauda*	This species usually live as colonies of 2-4 cells, rarely 8 cells. Cells are oval or cylindrical. Each end of outer cells has a long spine, sometimes with a short spine or a nod.
007	678	*Amphiprora alata*	Cells are usually encountered as solitary with thin valve. Cell valve surface is lozenge with the wing-shaped curve S. The girdle is figure 8 and two pigments are flattened.
005	652	*Chaetoceros muelleri*	Cells are usually solitary, sometimes form chain of 2-3 cells, with oval frustule. There are two long, small spines on each end and nearly parallel.
006	1623	*Gyrosigma limosum*	Cells have sigmoid valves, and lack septa and intercalary bands. They possess spoon-shaped valve ends.
009	-	*Lauderia* sp.	Cells are cylindrical with thin valve. Valves stick together to form linear chains.
008	350	*Melosira nummuloides*	Cells are cylindrical to subspherical, with high mantles, well developed girdles and usually form long chaius.
002	1745	*Navicula radiosa*	This species is unicelullar with solitary frustules and narrow valve. Lanceolate is gradually tapering from the middle to the acute ends. Axial area is indistinct and central area is somewhat rounded.
001	1359	*Navicula tuscula*	Cells can be solitary or standing two cells together. Axial area is broaden. There are two laminate chromatophores sometimes splitting up into numerous small rounded granules.
003	-	*Navicula* sp.	Cells have solitary frustules, linear and rectangular girdle. Valve is rhomboidal, lanceolate and obtuse at the produced ends with straight raphes.
019	-	*Nannochloropsis* sp.	Cells are spherical or slightly ovoid without flagellate. Each cell has a chloroplast, which does not bear a pyrenoid.

-: not determined

especially in BBM and f/2 (data not shown). BBM and f/2 were used for biomass culture of chlorophytes and the other strains, respectively for anticancer activity assays. All the glassware, pipettes and forceps that are used in isolations and culture work are sterilized to prevent bacteria contamination and interference to following activity assays. This is to ensure that it is the algae which produce the activity not the associated bacteria or fungi.

Anticancer activity of cultured microalgal strains: Screening of crude extracts plays an important role in the procedure of finding and developing new drugs. Cytotoxicity tests against various cancer cell lines are the most common screening methods for anticancer compounds (Umamaheswari and Govindan, 2007). Extracts of fifteen microalgal strains were evaluated for cytotoxicity against KB cells using MTT based assay. The result is shown in Table 2.

Fifteen tested strains showed various activities against KB cell growth. Some strains of *Scenedesmus* sp. were reported to have antibacterial activity and inhibition against the development of echinoderm eggs (Murakami *et al*., 1988) as well as activity against some tumour cell lines (Ordog *et al*., 2004) but the isolated strains in this study had no clear activity. The activity of *Chlorella* strains in this study was also at low level. *Chlorella vulgaris* was shown to have chemopreventive effect in induced liver cancer and breast cancer rats and possess a glycoprotein with antitumour effects (Sulaiman *et al*., 2006; Amin, 2009). The *Chlorella vulgaris* strain in this study had some inhibition to KB cells but at low level. Previous report emphasized the importance of strain selection as activity varied between strains of the same species (Ordog *et al*., 2004). *Ankistrodesmus gracilis* VACC-010 and *Amphiprora alata* VACC-007 exhibited strongest inhibition to KB cells (26.50 and 29.82 μg mL^{-1}, respectively) and thus were subjected to test against HepG2, SK-LU-1 and MCF7 cancer cell lines. Inhibitory concentration (IC_{50}) of each strain was shown in Table 3.

Amphiprora alata VACC-007 had no remarkable activity against these cell lines while *Ankistrodesmus gracilis* VACC-010 inhibited them all with the strongest activity against hepatic cancer cell line (HepG2) at the concentration of 9.64 μg mL^{-1}. The result suggested that *Ankistrodesmus gracilis* VACC-010 contain useful biological compounds and need further characterization. As they are relatively easily grown in mass culture with BBM medium, they can provide a potential source for anticancer pharmaceutical application.

Table 2: Activity of extracts of microalgal strains against KB cells

Microalgal strains	IC_{50} (μg mL^{-1})
Ankistrodesmus gracilis (VACC-010)	26.50
Chlorella vulgaris (VACC-012)	78.98
Chlorella sp. (VACC-013)	>128
Chlorococcum sp. (VACC-018)	>128
Scenedesmus quadricauda (VACC-014)	>128
Scenedesmus bijugatus (VACC-015)	>128
Amphiprora alata (VACC-007)	29.82
Chaetoceros muelleri (VACC-005)	>128
Gyrosigma limosum (VACC-006)	>128
Lauderia sp. (VACC-009)	>128
Melosira nummuloides (VACC-008)	>128
Navicula radiosa (VACC-002)	>128
Navicula tuscula (VACC-001)	67.24
Navicula sp. (VACC-003)	66.99
Nannochloropsis sp. (VACC-019)	40.25

Table 3: Activity of extracts of microalgal strains against three cancer cell lines

	IC_{50} (μg mL^{-1})		
Microalgal strains	HepG2	SK-LU-1	MCF7
Ankistrodesmus gracilis VACC-010	9.64	58.62	68.22
Amphiprora alata VACC-007	>128	>128	>128

CONCLUSION

In this study, fifteen microalgal strains were successfully isolated and characterized from mangrove in Xuanthuy National Park. Although, the extent of screening for anticancer activity is still modest, a potential candidate, *Ankistrodesmus gracilis* VACC-010 was found. It has effective inhibition against two tested cell lines. This is the first report on the anticancer activity of this strain as well as the first screening of mangrove microalgae for biologically active compounds. More diverse pharmacological evaluation of algal extracts and isolation of active compounds from *Ankistrodesmus gracilis* VACC-010 are underway.

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