DOI : 10.35124/bca.2019.19.2.4163

Biochem. Cell. Arch. Vol. 19, No. 2, pp. 4163-4167, 2019 | www.connectjournals.com/bca | *ISSN 0972-5075*

ALGICIDAL, CYTOTOXICITY AND ANTIOXIDANT PROPERTIES OF ALGICIDAL BACTERIA ISOLATED FROM DINOFLAGELLATE *GAMBIERDISCUS BELIZEANUS*

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(Received 14 April 2019, Revised 27 July 2019, Accepted 11 August 2019)

ABSTRACT : The study is aimed to evaluate the algicidal activity, antioxidant and cytotoxicity properties of the extract by ethyl acetate of algicidal bacteria *Loktanella* sp. Gb03 UKMGb03A (KU199217)). The algicidal activity of the ethyl acetate extract of *Loktanella* sp. Gb03 was assessed against toxic dinoflagellate *Coolia malayensis*. The cytotoxicity property was determined by MTT assay and antioxidant activity was detected by DPPH radical scavenging activity. The results of algicidal activity assay showed that the *Loktanella* sp. Gb03 extract possesses algicidal activity against toxic dinoflagellate *Coolia malayensis*. The *Loktanella* sp. Gb03 showed higher antioxidant activity compare to standard antioxidant ascorbic acid. In addition, *Loktanella* sp. Gb03 exhibited low cytotoxicity. However, the *Loktanella* sp. Gb03 has strong algicidal activity and antioxidant activities that are potent associated with its ethno medicinal values.

Key words : *Gambierdiscus belizeanus*, algicidal bacteria, antioxidant properties, cytotoxicity properties.

INTRODUCTION

The Interactions between harmful algal bloom (HAB) species and bacteria play an important rules of regulating of these algae. Harmful algal bloom (HAB) contaminate coastal life has seriously damage effect on aquaculture industries (Salman *et al*, 2011) as well as human health and environmental (Anderson *et al*, 2012; Wang *et al*, 2012). Every year, were reported around more than two thousand cases of human poisoning of algal toxins (Zingone and Enevoldsen, 2000). Coastal Harmful algal bloom toxin have been estimated to result in economic impacts in the United States of America more than $82 million per year. These impacts stress the importance of developing tools to avoid their impacts, as well as, understanding harmful algal bloom and control or prevent the algal blooms.

The marine bacteria is a rich source of bioactive molecules with unlimited functional diversity and chemical. Almost 30,000 natural products from marine organisms have been isolated, as well as, many of the drug candidates are in clinical trials (Salman *et al*., Zhang *et al*, 2011). In the studies reported recently, both, seafloors and deep sea water bodies have been shown to be unlimited source of bioactive compounds on the planet (Zingone and Enevoldsen, 2000). Microorganisms are an important sources of bioactive compounds with agricultural and pharmaceutical importance. There is a highly interest in the algicidal activity for the control of Harmful algal bloom (HAB). Algicidal bacteria extracts isolated from marine water are considered prolific resources for novel algicidal substances with various structures and new mechanisms of action.

This study were purposed to investigate algicidal activity by used 24-well plate and counting chamber, as well as, evaluate antioxidant activity by scavenging capability of DPPH free radical and to assess the possible cytotoxicity property of the ethyl acetate extract of *Loktanella* sp. Gb03.

MATERIALS AND METHODS

Culture condition of algicidal bacteria

Loktanella sp. Gb03 was isolated from dinoflagellate culture (*Gambierdiscus belizeanus*) in laboratory by serial dilution technique on nutrient agar. Strain Gb03 was maintained on nutrient agar at 4°C for short-term preservation and in a glycerol suspension (20%, w/v in distilled water) at -80°C for long-term preservation.

Preparation of extracts and its activity

In this experiment, culture of Gb03 strain in marine broth for 36 hours at 30°C were centrifuged at 15000xg for 15 minutes and followed by filtering the supernatant through 0.2 µm Millipore membranes, extraction from

filtrated supernatant by using organic solvents Ethyl acetate at ratio of 1:1 (v: v). The supernatant was mix with solvent (overnight) in separation flask. The solvent was evaporated until minimum amount, and was drying for three days (Ezema, Eze and Ezeofor). The dried extract was dissolved in DMSO for further study.

The algicidal activity of strain Gb03 was tested against *C. malayensis* (1.0×10^3 cells/mL) used 24-well plate. Each well contain 1mL of *C. malayensis* culture, to which 10% (V: V) from the prepared solution were added. The plates were monitored at magnification of ×100. The plates were inspected after 24 hours incubation time in alga condition (light intensity of 140 ìmol $m^{-2}s^{-1}$ and 12:12 hours light : dark photoperiod).

Determination of the cytotoxic activity of *Loktanella* sp. Gb03 crude extract

In order to examine the cytotoxicity assay of crude extract of *Loktanella* sp. Gb03 on the viability of Vero cell (African green monkey kidney cells). MTT assay was carried out corresponding to technique prescribed by Raheel *et al* (2013). Vero cells line was collected from the stock compilation of the virology lab, school of Biosciences and Biotechnology, Faculty of Technology, UKM. The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Sigma- Aldrich, USA) supplemented with 5% Foetal Bovine Serum (FBS) as grow media. Cultures for the assay were made from confluent monolayer layer cells, planted at a density of 2×104 cells/well in 96-well microplate's flat bottom and incubated at 37°C overnight in a 5% CO_2 to assist adherence of the cells. The growth medium on the confluent Vero cells growth in few hours was removed and exchanged with 100 µL(100 µg/mL) of bacterial crude extract. It was preceded by replacement in 5% Dimethylsulfoxide (DMSO) and preparation in growth medium at different concentrations. The vitality of cells was analyzed by MTT assay based on the reductive cleavage of the 3-(4, 5—2-yl)-2, 5-di Dimethylthiazolphenylte trazolium bromide (yellow tetrazole) after incubation at 37°C in a 5% CO_2 for 48h, by mitochondrial dehydrogenase enzyme present in live cells to produce purple formazan crystals. The medium was discarded, 100 µL of DMEM and 20 µL of 5 mg/mL MTT dissolved in Phosphate Buffer Solution (PBS) were introduced to each well. The plates were incubated again for 4 hours, kept under similar environment. MTT was discarded and 100 µL of DMSO was introduced into every well. Later, the plates were lightly shaken to dissolve the formazan crystals. Berberine chloride (Sigma, France) was utilized as positive control. The optical density (OD) of every well was calculated at wavelength of 540 nm by an ELISA reader (CDS, India). Cytotoxicity was expressed as 50% Cytotoxic Concentration (CC50) of components that prevent the groeth of cells by 50% in contrast to untreated cells. The percentage of cell vitality was calculated as given below:

$$\text{Viability} = \frac{(\text{Treated cells} - \text{Blank})}{(\text{Untreated cells} - \text{Blank})} \times 100$$

For the purpose of measuring Selectivity Index (CC_{50}), selective index of crude extract was calculated by used Graph Pad Version 7.

Determination of the Antioxidant activity of *Loktanella* sp. Gb03 crude extract

Quantitative evaluation of antioxidant action was carried out on the *Loktanella* sp. Gb03 crude extract utilizing a 2,2-diphenyl-1- picrylhydrazyl (DPPH) assay as described by Sunil, Agastian *et al* (2012). The free radical capabilities were founded spectrophotometriclly against DPPH (DPPH, Sigma, France). Antioxidant element can provide hydrogen that reacts with DPPH. At 517 nm on a UV/visible light spectrophotometer, the alterations in color (from deep violet to light yellow) were measured (SpectropicGenesys 8, Rochester, USA). The antioxidant action was analyzed DPPH scavenging method. By dissolving 40 mg DPPH in 100 µL methanol, the stock solution was collected which was preserved at 20°C for later use. Nearly 350 µL stock solution was added with 350 µL methanol to collect the absorbance of 0.70±0.01 unit at 517 nm wavelength with the help of spectrophotometer (Epoch, Biotek, USA). 100 mg of dry extract dissolved in 1 mL methanol, serial dilutions 0.175, 0.490, 0.811, 1.622, 3.455, 6.235, 12.775, 24,650 and 100.123 µg/mL of crude extract were made. 100 µl of Trolox were add as a reference triplicate for everyone from these concentration (0.175, 0.490, 0.811, 1.622, 3.455, 6.235, 12.775, 24.650 and 100.123 µg/mL as a blank then add 1000 µl DPPH). Keep them at dark area was rest for few hours and calculated them with 516 nm. (Sunil, Agastian et *al*, 2012). The percentage of DPPH scavenging action was determined according to the equation:

$$\text{DPPH scavenging activity (\%)} = \frac{(\text{A blank} - \text{A sample})}{\text{A blank}}$$

Where, A is the absorbance at 516 nm.

For the purpose of measuring Selectivity Index (CC_{50}), selective index of *Loktanella* sp. Gb03 crude extract was calculated by used Graph Pad Version.

RESULTS

Preparation of extracts and its activity

Among the tested extracts (Methanol, Hexane, ethyl acetate, Chloroform, Acetonitrile and water), the ethyl acetate extracts of *Loktanella*sp Gb-03 showed higher activity against toxic dinoflagellate *C. malaynesis*. The results of Ethyl acetate and other solvents extracts (Methanol, Hexane, ethyl acetate, Chloroform, Acetonitrile and water) were 32%, 74%, 100, 78%, 77% and 11% respectively (Fig. 1).

Cytotoxicity effects of marine *Loktanella* sp. Gb03 crude extracts

The Half Maximum Cytotoxicity Concentration (CC_{50}) value of *Loktanella* sp. Gb03 crude extracts was 79.4 µg/mL shown in the graph (Fig. 2). The resulted showed that the cytotoxicity effects of the *Loktanella* sp. Gb03 crude extracts were increased with the increase of the extracts concentration. According to the American National Cancer Institute (NCI), guidelines set the limit of activity for crude extracts at 50% inhibition (CC_{50}) of proliferation of less than 30 ìg/mL after the exposure time of 72 hours (Abdel-Hameed *et al*, 2012). However, a crude extract with CC_{50} less than 20 µg/mL is considered highly cytotoxic (Mahavorasirikul *et al*, 2010). The results of the present study showed not cytotoxic effects on Vero cells with *Loktanella* sp. Gb03 crude extracts at CC_{50} equal to 79.4 µg/mL.

DPPH radical scavenging activity of *Loktanella* sp. Gb03 ethyl acetate extract

The free radical scavenging effect of the ethyl acetate extract of *Loktanella* sp. Gb03 was performed by using DPPH. It is possible to determine antioxidant ability by plotting percentage inhibition of the DPPH radical as a task of *Loktanella* sp. Gb03 ethyl acetate extract and Trolox in mg/mL. Error bars (calculated from repetitions occurring thrice by used Graph Pad Prism 7) exist on graphs.

Fig. 3 (a) and (b) showed the scavenging effect of *Loktanella* sp. Gb03 ethyl acetate extract was higher than that of Trolox and half-maximum concentration (IC_{50}) of *Loktanella* sp. Gb03 ethyl acetate extract and positive control (Trolox) were observed in values 6.131 and 6.6039 µg/mL, respectively. These result indicated that *Loktanella* sp. Gb03 ethyl acetate extract had prominent antioxidant activity equivalent to positive control Trolox.

DISCUSSION

The isolate Gb03 produced algicidal activity against dinoflagellate, this algicidal activity was hypothesised to be the result of the production of an algicidal molecule being produced by the bacteria. This molecule was extracted via ethyl acetate from the algicidal bacterial Gb03. There is no clear depiction of the algicidal activity of *Loktanella*sp. Gb03, hence purification of the extract was needed. The algicidal bacteria *Loktanella*sp. Gb03 extracted with six different organic solvents (Methanol, Ethyl acetate, Hexane, Chloroform, Acetonitrile and water), to understand the polarity of the active compounds and also to ascertain which solvent systems would be suited in extraction. However, there are many algicidal compounds reported, which isolated by different solvents, for example, *Alteromonas* sp. KNS-16 isolated from a harmful algae bloom area which found to control HABs by producing compounds in an indirect way, the algicidal compounds of algicidalstrain KNS-16 was isolated by Acetone (Cho, 2012). Algicidal bacteria Pseudomonas K44-1 were isolated from the surface water (Tokyo, Japan) showed marked antialgal activity by producing algicidal compounds which extracted by ethyl acetate us solvent (Kodani *et al*, 2002). The algicidal compounds of algicidal bacteria hewanella sp. Lzh-2, isolated by ethyl acetate as solvent (Li *et al*, 2014). This suggested that the compounds responsible for the algicidal activity could be a hydrophobic compounds. Furthermore, there are various antimicrobial compounds that have varying hydrophobicity, it can be highly hydrophobic (Evers *et al*, 2008; Raaijmakers *et al*, 2006) or low hydrophobic (Fedeniuk and Shand, 1998).

Cytotoxicity effects of marine *Loktanella* sp. Gb03 crude extracts

In vitro cytotoxicity bioassay is considered as an important method for preliminary evaluation of cytotoxicity of different compounds and extracts because it useful, rapid, efficient and inexpensive. The cytotoxicity assay of the secondary metabolite of *Loktanella* sp. Gb03 crude extracts was evaluated on monkey kidney of the Vero cells by 3-(4, 5-dimethylthiazol- 2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

The CC_{50} value was found to be higher than that specified by NCI. The reduction in viable cell number was evident as 72 hours of treatment with the extracts at the concentration after 79.4 µg/mL. According Alwash (2014) studies showed that Methanol extract of *M. malabathricum* leaves (MMML) is not cytotoxic to Vero cells (CC_{50} >30 µg/mL) (Alwash *et al*, 2014). Also, Vijayarathna and Sasidharan (2012) reported that the cytotoxicity effect of *Elaeis guineensis* extract had the cytotoxicity effect due to inhibit the proliferation of the Vero cells at (IC50 22 µg/mL) (Vijayarathna and Sasidharan, 2012). The investigation provides evidence

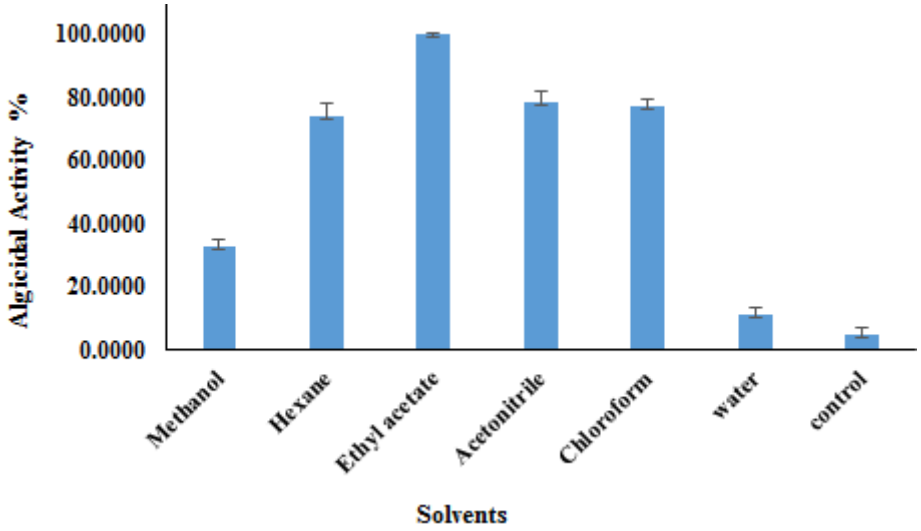

Fig. 1 : Algicidal activity of different solvents extract on *C. malaynesis*, while using nutrient broth medium extractby ethyl acetate as a control and DMSO as a second control.

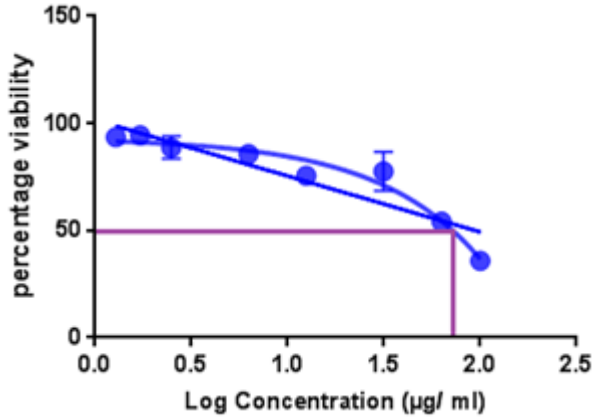

Fig. 2 : Cytotoxicity assay of *Loktanella* sp. Gb03 crude extracts showed percentage viability of Vero cells using (3- (4, 5-dimethylthiazol- 2-yl) -2, 5-diphenyl-tetrazolium bromide) MTT. The graphs constructed by plotting the viability percentage of the Vero cells against various concentration of crude extracts and error bars (calculated from three replicates by used Graph Pad Prism 7) are shown in the graphs
Log CC_{50} = 1.91 (IC_{50} = 79.432823 µg/mL)

for anti-cytotoxicity in AFEA, which may be due to existing chemicals in the extract since *Alcaligenes faecalis* as mention previously.

Antioxidant activity study

Antioxidant activity compounds are any chemicals compound which has the ability to block the activity other chemicals compound known as free radical. Antioxidant compounds have many properties, of which an important one is its action of capturing free radicals. Free radicals which be highly reactive and oxygen species are existing in many biological systems from a wide variety of sources. These free radicals have the ability oxidize nucleic acids, proteins, lipids or DNA and can result in degenerative illnesses. Reactive free radical possess the ability to

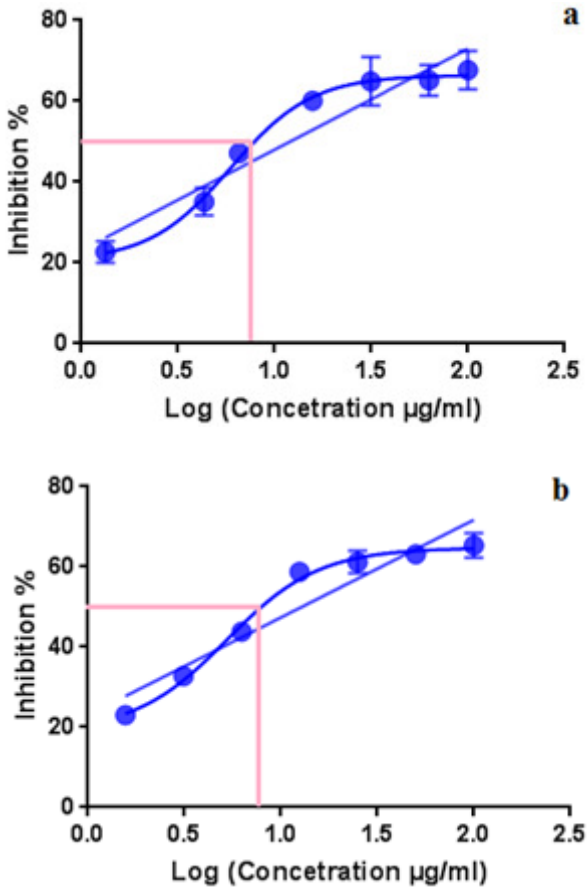

Fig. 3 : Inhibition percentage DPPH versus concentration of Trolox (a) and *Loktanella* sp. Gb03 ethyl acetate extract (b). The graphs constructed by plotting the inhibition percentage of DPPH against various concentration of *Loktanella* sp. Gb03 ethyl acetate extract and error bars (calculated from three replicates by used Graph Pad Prism 7) are shown in the graphs
LogIC_{50} of Trolox = 0.8289 (IC_{50} = 6.6039 µg/mL)
LogIC_{50} of *Loktanella* sp. Gb03 ethyl acetate extract = 0.7876 (IC_{50} = 6.131 µg/mL).

cause damage to cell, and is carcinogenic (Storz, 2005). Antioxidant substances scavenge free radicals which are peroxide, hydro peroxide and lipid peroxyl and put an end to oxidative processes, which lead to degenerative illnesses. Various types of methods exist in the literature pertaining to antioxidant abilities, out of these analytical process, a specific one computes the radical scavenging ability of antioxidant in opposition to free radicals such as 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, the superoxide anion radical (O_2), the hydroxyl radical (OH), or the Peroxyl radical (ROO).

In the current study, the method of scavenging which used of the DPPH radical is one of the wide methods was used. The antioxidants effect on scavenging DPPH radical is because of their ability for hydrogen donating. DPPH radical has an absorption band set at 516 nm and is a stable free radical. It gets devoid of this property when gaining free radical species or an electron, which results in a visually noticeable discoloration from purple to yellow. Many samples can be included in a small time frame and it has the ability to recognize crude compounds activity at reduced levels of concentrations (Hseu *et al*, 2008). The reduction in absorption of DPPH shows that crude extraction compounds can scavenge free radicals without help of enzymes (Lebeau *et al*, 2000).

Similar results were reported in earlier studies (Hseu *et al*, 2008; Lam, 2006). Where the crude extract showed highest radical scavenging potential, it is obvious that the AFC showed hydrogen donating ability and therefore the extract could serve as free radical scavengers, acting possibly as primary antioxidants.

CONCLUSION

The *Loktanella* sp. Gb03 showed higher antioxidant activity compare to standard antioxidant ascorbic acid. In addition, *Loktanella* sp. Gb03 exhibited low cytotoxicity. However, the *Loktanella* sp. Gb03 has strong algicidal activity and antioxidant activities that are potent associated with its ethno medicinal values.

ACKNOWLEDGMENTS

The authors thank Dr. Fareed Sairi for his contributions to this work. This work was supported by grants 04-01-SF14 and UKM GUP BTK 07-75-198.

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