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ฤทธิ์ต้านแบคทีเรียและต้านอนุมูลอิสระของสาหร่าย Nostoc commune Vaucher ex Bornet & Flahault

Antibacterial and antioxidant activities of Nostoc commune Vaucher ex Bornet & Flahault

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Abstract

Pathogenic bacteria from food contamination are causative agents of enteric diseases. Antibacterial agents may cause toxicity and side effect after treatment. Moreover, drug resistant bacteria may emerge after long term of drug usage. Thus, the use of natural extracts is an alternative choice for treatment of enteric diseases. In this study, antibacterial and antioxidant activities of *Nostoc commune* extracts were investigated. Aqueous and ethanolic extracts of *N. commune* were tested against Salmonella Typhimurium, *Enterobacter aerogenes*, and *Proteus vulgaris* by agar well diffusion and broth dilution methods. It was found that ethanolic extract of *N. commune* showed the highest anti-bacterial activity on *E. aerogenes* with MIC of 15.62 mg/mL and MBC of 250 mg/mL. Moreover, the antioxidant activity of extracts was determined. The results showed that antioxidant activities of ethanolic extract from *N. commune* were higher than aqueous extract in all models of antioxidant test. Antioxidant activities of ethanolic extract of *N. commune* that were determined

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by metal chelating assay, the reducing power, ABTS radical cation decolorization and DPPH$^{\bullet}$ radical scavenging assay were 0.533 mg EDTAE/g extract, 0.513 mg GE/g extract, 3.064 mg TE/g extract and 0.013 mg GE/g extract, respectively. Moreover, the total contents of phenolics and flavonoids were determined. It was found that the ethanolic extract of *N. commune* showed total phenolic and flavonoid content of 0.482 mg GE/g extract and 1.1 mg QE/g extract. The knowledge obtained from this study will be useful in order to develop therapeutic potential agent from *N. commune* as antioxidant in food supplement and antibacterial agent in the future.

Keywords: *Nostoc commune*, antibacterial, antioxidant

1. INTRODUCTION

Free radicals were found to be a product of normal metabolism in the body. The free radicals can damage and cause molecular and gene mutations leading to several disease conditions [1]. Many researchers have become more interested in natural source which could block or reduce free radicals [2, 3, 4]. Senthilkumar and Sudha [5] have reported that extract of green seaweed *Chaetomorpha linum* has potential as a natural antioxidant and a natural source of antimicrobial agents against many microorganisms. Antioxidant activity of polysaccharide from *Nostoc sphaeroides* was investigated, and the polysaccharide had effective activity on scavenging of reactive oxygen species (ROS) *in vitro* [6].

Nostoc is a genus of nitrogen-fixing cyanobacteria, family Nostocaceae. It has the ability to use atmospheric nitrogen when combined nitrogen is not available [7]. *Nostoc* can form spherical and large gelatinous macrocolonies. It also composes of filaments, which is called trichomes surrounded by thin sheath [8]. *Nostoc* can be found in various habitats such as soil, moist rocks, brackish water and paddy field [9]. Cytotoxic agents such as noscomin and muscoride were isolated from *N. commune* and *N. muscorum*, and the compounds have been shown as novel antibacterial agents [10, 11]. Moreover, *N. spongiaeforme* var. *tenue* produced antibacterial and cytotoxic agents [12]. Antiviral activities were also reported from the extract of *Nostoc flagelliforme* [13]. In Thailand, *N. commune* is widespread as cyanobacterial macrocolonies. It is commonly known as Hed Lab and can be used as food source for local people in northern and north-eastern parts of the country [14, 15]. In China, this species have been used as a food source or medicine to treat illness [16]. Therefore, the aim of this research was to investigate the antibacterial activity of ethanolic and aqueous extracts of *N. commune* against three pathogenic bacteria in digestive system; *Proteus vulgaris*, *Enterobacter aerogenes* and *Salmonella typhimurium.* Antioxidant activity of *N. commune* extracts was also evaluated *in vitro* by 6 models of antioxidant tests.

2. MATERIALS AND EXPERIMENTS

2.1. Algal extract preparation

Nostoc commune Vaucher ex Bornet & Flahault was collected in April 2014 from Klongthom district, Krabi province, Southern Thailand. For morphological analysis, cell dimensions including length and width of vegetative cells, heterocysts and akinetes of filaments were studied using a Nikon e200 light microscope. Taxonomic identification was based on morphological characteristics presented in standard literature [8, 9]. The samples of *N. commune* were washed in distilled water, dried and milled to obtain dried powder. The dried powder was extracted with ethanol and water by maceration. The extracts were filtered through filter paper Whatman No. 1. The solvent was evaporated and the filtrate was concentrated by rotary evaporator. Next, the extracts were lyophilized to obtain dried powder and were dissolved in 10% dimethyl sulfoxide (DMSO) to prepare the solution of extract before use. The stock concentration of *Nostoc* extract was 1,000 mg/mL.

2.2. Bacterial strains

Salmonella Typhimurium, *Enterobacter aerogenes*, and *Proteus vulgaris* were obtained from Faculty of Liberal Arts and Science, Kasetsart University Kamphaeng Saen Campus, Nakhon Pathom, Thailand.

2.3. Antimicrobial bioassay

2.3.1 Agar well diffusion assay

Agar well diffusion assay was modified from the previous study by Perez *et al* (1990) [17]. Bacteria were cultured in Muller Hinton broth (MHB) at 37°C for 24 hours and then adjusted to final inoculums approximately 10^8 CFU/mL. The inoculums were swabbed on Muller Hinton agar (MHA) and 6 mm diameter wells were performed on agar plates using sterile cork borer. *Nostoc commune* extract (30 µL) at concentrations of 250, 375, and 500 mg/mL were added into wells. The plates were incubated at 37°C for 24 hours. The antibacterial activity of extract was determined in terms of mean of inhibition zone. The diameter of inhibition zone was measured. Ceftriazone and DMSO were used as positive and negative control, respectively.

2.3.2 Assessment of Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC)

The MIC and MBC of active extracts were measured by broth dilution method [18]. Algal extract was two-fold diluted in MHB. Then, 0.5 mL of bacterial inoculum (10^5 CFU/mL) at the highest concentration (1,000 mg/mL) was added into the first tube and two-fold serial dilution was performed by transferring 0.5 mL of suspension to the subsequent tubes. The final tube of suspension was discarded. Standard bacterial suspension at 10^8 CFU/mL was added to each dilution. Bacterial inoculum in culture broth was

used as positive control and broth was used as negative control in the experiment. Ceftriazone were used as positive control. The test tubes were then kept at 37°C for 24 hours. MIC was defined as the lowest concentration of extracts that inhibited the growth of bacteria. For MBC determination, 0.01 mL of culture broth in the tubes with no growth was streaked on MHA and cultured at 37°C for 24 hours. MBC was defined as the lowest concentration that inhibited growth of bacteria by 99.9%.

2.4. Antioxidant activities

2.4.1 ABTS radical cation decolorization assay

The scavenging activity of $ABTS^{\bullet+}$ radical activity was performed according to the modified method of Re *et al* [19]. The antioxidant activity of ethanolic and aqueous extracts was determined by decolorization of ABTS [2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)] radical cation. ABTS radical cation (Blue green color) was firstly prepared by oxidation of 5 ml of 7 mM ABTS with 88 µl of 140 mM potassium persulfate in water and kept in the dark at room temperature for 12-16 hours before use. Absorbance of ABTS radical cation solution at 734 nm was 0.700 ± 0.50 and it was used as absorbance of control. Trolox (Sigma-Aldrich) was used as antioxidant standard. Various concentrations of extracts (10 µL) were added into 1 mL of ABTS radical cation reagent and the mixture was incubated in the dark for 6 minutes. The absorbance of ABTS radical after treatment with plant extracts at 734 nm was measured. In this study, antioxidant activity was expressed as mg of trolox equivalents (TE) per 1 gram of sample.

2.4.2 DPPH radical scavenging activity

2,2′diphenyl-1-picrylhydrazyl (DPPH) radical scavenging of extracts was measure by DPPH assay according to the modified method of Hoe *et al* [20]. Extract (800 µL) at various concentrations was mixed with 67 µL of 1 M Tris-HCl (pH 7.9) and 800 µL of 130 µM DPPH in methanol. The reaction mixture was kept in the dark at room temperature for 20 minutes and the absorbance was measured at 517 nm. Gallic acid was used as a standard. The decrease of absorbance at 517 nm was determined. Percentage inhibition was calculated as follows: $[(A_{control} - A_{sample}) \times 100 / A_{control}]$. This assay was expressed as mg of gallic acid equivalents (GE) per 1 g of extract.

2.4.3 Reducing power assay

Reducing power of crude ethanolic and aqueous extracts obtained from *N. commune* was determined according to the modified method of Oyaiza [21]. Sample (120 µL) was mixed with 290 µL of 0.2 M phosphate buffer (pH 6.6) and 290 µL of 1% w/v potassium ferricyanide. Reaction mixture was incubated at 50°C for 20 minutes. Then, 10% TCA (290 µL) was added and centrifuged at 3000 X *g* for 10 minutes. Supernatant (1 mL) was mixed with 1 mL of deionized water and 0.2 mL of 0.1% $FeCl_3$. Absorbance of the sample solution was measured at 700 nm and calculated as percentage of inhibition, and expressed as GE/ g of extract.

2.4.4 Metal chelating activity

The metal chelating effect on ferrous ions of extracts was determined according to the modified method of Dinis [22]. *Nostoc* extract (400 µL) was mixed with 25 µL of 2 mM $FeCl_2$ and 100 µL of 5 mM Ferrozine. The reaction was then left at room temperature for 10 minutes and then absorbance was measured at 562 nm. The percentage of inhibition of ferrozine complexs with Fe^{2+} was calculated as follows: $[(A_{control} - A_{sample}) \times 100 / A_{control}]$. EDTA was used as a control. This assay was expressed as ethylenediaminetetraacetic acid equivalent (EDTAE), which defined as mg of EDTAE per g of extract.

2.4.5 Determination of total phenolic content

Total phenolic content was determined by Folin-Ciocalteu method [23]. Extract (250 µL) was mixed with 1.25 mL of deionized water. Then, 250 µL of 95% ethanol and 125 µL of 50% Folin-Ciocalteu were added. After incubation for 5 minutes, 250 µL of 5% sodium carbonate was added and incubated at room temperature for 1 hour. The absorbance was measured at 725 nm. Gallic acid was used as a standard. The content of phenolic compound was expressed as mg of GE per 1 g of extract.

2.4.6 Determination of total flavonoid content

The flavonoid compounds were determined by aluminum chloride colorimetric assay[24]. Extract (250 µL) was added to 50 µL of 10% aluminum chloride. Then, 750 µL of methanol, 50 µL of 1M potassium acetate and 1.4 mL of deionized water were mixed. The mixture was incubated at room temperature for 30 minutes in the dark. The absorbance was measured at 415 nm. Quercetin was used as positive control. All tests were carried out in triplicate. Total flavonoid content was expressed as mg quercetin equivalent (QE)/ g extract.

2. 5 Statistical analysis

All experiments were carried out in triplicates. Data values were expressed as mean ± standard deviation.

3. RESULTS AND DISCUSSIONS

3.1 Antibacterial activities

S. Typhimurium, *E. aerogenes*, and *P. vulgaris* are Gram negative bacteria and can cause human illness in developing countries. The bacteria are a causative agent of diarrhea and commonly responsible for infections in hospitals. Outbreak causes by S. Typhi resistant strains have been reported in many developing countries especially India, Southeast Asia and Africa [25]. Although, effective chemically synthesized drugs have been used to treat many diseases but the drugs have side effects and are quite expensive. Therefore, natural products have been the most productive source and provide active ingredients for the development of drugs. Thus, natural-product-based drugs are an alternative choice in recent year.

In this study, aqueous and ethanolic extract of *N. commune* were evaluated for antibacterial activity by agar well diffusion assay. MIC and MBC values were also determined. *N. commune* extracts concentration at 250, 375, and 500 mg/mL were used in this study. The results showed that the ethanolic extract of *N. commune* showed inhibitory effect against different bacterial species whereas aqueous extract did not show the activity against *P. vulgaris, E. aerogenes* and S. Typhimurium. The solvent (10% DMSO) could not inhibit growth of bacteria. 100 mg/mL of ceftriazone could be inhibit S. Typhi with inhibition zone of 40.8 mm. Results were presented in Table 1.

TABLE 1: Inhibition zones of ethanolic and aqueous extracts of *N. commune* against *P. vulgaris, E. aerogenes,* and S. Typhimurium

Type of extract	**concentration**	**Zone of inhibition* (mm)**		
	(mg/mL)	***P. vulgaris***	***E. aerogenes***	***S. typhimurium***
Aqueous	250	0	0	0
	375	0	0	0
	500	0	0	0
Ethanol	250	0	6.6 ±0.76	0
	375	3.0 ±0.65	8.0 ±0.58	1.0 ±0.55
	500	3.6 ±0.22	9.3 ±0.01	2.0 ±0.14
Ceftriazone	25	ND	36.1 ±1.05	34.8 ±0.25
	50	ND	36.3 ±0.78	35.1± 0.41
	100	ND	38.3 ±2.81	40.8 ±0.17

Data in table are given as mean ± standard deviation from triplicate experiments.

*Zone of inhibition = clear zone of test – clear zone of 10% DMSO control

ND = not detectable

The ethanolic extracts of *N. commune* at 500 mg/mL showed the most effective activity against *E. aerogenes* with inhibition zone of 9.3 mm. Moreover, ethanolic extract of *Nostoc* showed very low activity against *P. vulgaris* and S. Typhimurium. Therefore, the MIC and MBC values of ethanolic extract of *N. commune* against *E. aerogenes* were evaluated using broth dilution method. The ethanolic extract was able to inhibit the growth of *E. aerogenes* with the MIC and MBC values of 15.62 and 250 mg/mL. Interestingly, the ethanolic extract of *N. commune* could inhibit growth of Gram negative bacteria, which has complex cell wall structure more than Gram positive bacteria. Cell wall in Gram negative cells consists of outer membrane that covers a thinner

layer of peptidoglycan. Lipopolysaccharides confer their net negative charge to Gram negative bacteria [26]. Thus, it became impermeable to antimicrobial substance. Hence, the results showed that *N. commume* might be promising natural source of antimicrobial properties to be developed as therapeutic antibacterial agent for Gram negative bacteria, specifically *E. aerogenes*.

Antibacterial agents from herbs and algae have been reported in many studies. *Salvia officinalis* L. essential oils showed inhibitory effect on *E. coli, S.* Typhi*, S.* Enteritidis and *Shigella sonei* [27]. Antimicrobial activity of ethanolic extract from *Salvia trichoclado* L. was reported against *E. aerogenes* ATCC 13048 [28]. Aqueous, acetone, methanol and hexane extracts of *Anabaena* sp. showed specific inhibition effect on *Staphylococcus aureus* [29]. The ethanolic extracts of cyanobacteria, *Phormidium* sp. and *Microcoleus* extract were determined for antibacterial activity against *E. coli*. It was found that 0.2 g/mL of *Phormidium* sp. and *Microcoleus* sp. demonstrated effective antibacterial activity with diameters of inhibition zones of 10-11 mm [30]. As far as antimicrobial activity is concerned, *Nostoc linckia* was found to be most potent against *P. vulgaris* whereas MIC and MBC values of extract against *P. vulgaris* were 0.625 and 0.77 mg/mL, respectively [31]. The results showed that the differences of antibacterial activity of algal extracts might be attributed to the difference procedures for testing antibacterial activity. Differences in environment, climate, altitude, age and cultivar types and variety of algal extract may affect the antibacterial activity [32].

3.2 Antioxidant activities

Recently, many researches are focused to find natural antioxidant agents for use in food or novel pharmaceutical and to replace synthetic antioxidant agent. In this study, ethanolic and aqueous extracts of *N. commune* were investigated using 6 models of antioxidant activity. These methods differ in terms of their assay principles and experimental condition. The methods have varies contribution to total antioxidant potential [33, 34]. The effect of extracts was summarized in Table 2.

Table 2: Antioxidant activities of ethanolic and aqueous extracts of *N. commune*

Method	Antioxidant activity	
	Ethanolic extract	**Aqueous extract**
ABTS decolorization assay (mg TE/ g of extract)	3.064 ±0.81	1.678 ±0.12
DPPH radical scavenging (mg GE/g of extract)	0.013 ± 0.005	0.005 ±0.0001
Reducing power (mg GE/g of extract)	0.513±0.01	0.198±0.006
Metal chelating (mg EDTAE/g of extract)	0.533±0.03	0
Total phenolic content (mg GE/g of extract)	0.482±0.09	0.064±0.002
Total flavonoid content (mg QE/g of extract)	1.1 ±0.14	0

Data in table are given as mean ± standard deviation from triplicate experiments.

ABTS radical scavenging assay involved the generation of $ABTS^{\bullet+}$ by potassium persulfate. The extracts could reduce ABTS radical cation by acting as free radical scavengers or by hydrogen-donating to the molecule, which was measured at 734 nm. In the present study, the scavenging effect of 3.064 mg TE/g extract was shown by ethanolic extract. Furthermore, polyphenolics in plants and algae exhibited potential free radical scavengers or reducing agents [35, 36]. The aqueous extract of *Turbinaria conoides* and *Sargassum binderi* showed 0.036 and 0.024 trolox equivalent antioxidant capacity (TEAC) by ABTS radical scavenging activity, which was lower than antioxidant activity from *N. commune* in this study [37].

DPPH radical scavenging assay indicated the electron donation ability of extracts and could be measured by $DPPH^{+}$ solution bleaching. In this study, the ethanolic extract of *N. commune* showed DPPH radical scavenging activity of 0.013 mg GE/ g extract. Thus, the ethanolic extracts showed levels of DPPH scavenging higher than aqueous extract. Similarly, ethanolic extracts of *Halimeda macroloba*, *Sargassum binderi* and *Turbinaria canoides* showed dose dependent reduction of DPPH radical scavenging activity [37].

In reducing power, the presence of antioxidant substances caused the reduction of Fe^{3+}/ ferricyanide complex to ferrous form. They were responsible for reducing capacity that involved decomposition of peroxides radical scavenging and prevention of chain initiation [38, 39, 40]. Ferric

reducing power of ethanolic extract of *N. commune* was found to be 0.513 GE/ g extract. Similar observation was also reported that *Nostoc linckia* expressed ferric reducing power of 1.05 mg butylated hydroxy anisoles equivalent per gram extract [31]. Many studies investigated reducing power of algae and plant extracts [41, 42].

Reactive oxygen species are generated by products during metabolic reaction or mitochondrial electron transport. They are reformed as necessary intermediates of metal ion Fe^{2+} and possess the ability to continue free radical formation by obtaining or loss of electrons. Consequently, the reduction of ROS formation can be executed by metal ion chelating with chelating agents [43]. The present study, metal ion chelating method was carried out to estimate chelating power of extracts of *N. commune*, which demonstrated that the chelating capacity of ethanolic extract was 0.533 mg EDTAE/ g extract whereas aqueous extract had no effect on the chelating activity. Metal ion chelating contributed to oxidative damage in neurodegenerative disorder especially Alzheimer´s and Parkinson´s diseases [43, 44]. Hence, the result suggested that transition metal ion by ethanolic extract of *N. commune* was proved to be therapeutic importance.

Phenolic compounds were considered to be secondary metabolites and known to exhibit health promoting activities as antioxidant agents including phenolic acid and flavonoids [45, 46, 47]. The phenolic compound may be responsible to the possible antioxidant activity of many natural sources especially, plants and algae. Flavonoid was used in food industry because they blocked oxidative degradation of lipid. Moreover, hydroxyl groups conferred scavenging ability. Hydroxyl radical was one of ROS potency in the biological system, which caused damage to cell and initiated lipid peroxidation. In this study, the highest phenolic content was found from ethanolic extract with the value of 0.482 mg GE/g extract.

Many flavonoids have been discovered in the attractive colors of flowers, fruits and leaves [42, 46]. Flavonoids have been shown to have antimicrobial, anticancer, anti-allergic activity and prevent heart disease [40, 48, 49]. Flavonoid content of extracts was determined by aluminum chloride colorimetric assay. Moreover, total phenolic and flavonoid contents were observed in green algae, *Chlorella marina*, *Desmococcus olivaceous*, *Chlorococcum humicola* and *Dunaliella salina* and marine algae, *Porphyra* sp., *Navicula clavata*, *Sargassum wightii* Greville, *Chaetomorpha linum* (Muller) KÜtzing, and *Grateloupia lithophila* Boergesen [34, 39, 41, 50].

In this study, the results revealed that ethanolic extract of *N. commune* showed the highest amount of total flavonoid content of 1.1 mg QE/ g extract. Many studies have shown that selected phenolic and flavonoid compound possessed antimicrobial, anticancer and antioxidant activities [51, 52]. Studies on natural plants, vegetable and fruits have indicated the presence of

total phenolic and flavonoid content [40, 53, 54, 55]. China terrestrial algae compounds, β-ionone derivatives, nostocionone and 3-oxo-β-ionone were isolated form *N. commune* Vauch, which demonstrated strong antioxidant activities [56].

4. CONCLUSION

Ethanolic extract of *N. commune* showed the highest antimicrobial and antioxidant activities. The high amount of total phenolic and flavonoid content were also observed. The result from this study demonstrated the antimicrobial and antioxidant activity of *N. commune*. It also indicated the potential for development of an alternative source for used as antioxidant in food supplement and antibacterial agent.

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