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Editorial

NPC-Bromo: Special Issue

I am very grateful to Prof. Bambang Prajogo, Chairman, Bromo Conference (Symposium on Natural products & Diversity), and Dr. Tutik Sri Wahyuni, Department of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia, and the Organizing Committee for arranging this issue, originating from the Bromo Conference-2018, which was held in Surabaya, Indonesia, from July 11–12, 2018, and attended by a large number of participants.

The first part of the December 2018 edition is devoted to selected manuscripts (8) presented at Bromo-2018. I am very grateful to Profs. Bambang Prajogo and Tutik Sri Wahyuni for extending an invitation to participate in this scientific meeting, as well as for organizing this issue. The editors join me in thanking Profs Prajogo and Wahyuni, the authors, and the reviewers for their efforts that have made this issue possible, and to the production department for putting it into print.

Pawan K. Agrawal
Editor-in-Chief
Introduction to NPC Bromo Issue

This special issue contains selected papers previously presented at the Bromo Conference: Symposium of Natural Products and Biodiversity held in Surabaya on July 11-12, 2018. This symposium was organized by Universitas Airlangga, Surabaya, Indonesia in collaboration with the Indonesian Association of Natural Drug Researchers (PERHIPBA) and the Phytochemical Society of Asia (PSA). It was held to commemorate the 10th anniversary of the IOCD seminar in Surabaya. The Bromo Conference provides a forum for the exchange of information on Natural Products within all of the related topics, as well as with the aim to build and strengthen scientific cooperation between the research institutions.

Academic and other researchers, industrial practitioners and students participated in the symposium. The topics of interest covered in the Bromo Conference included ethnomedicine, implementation of the Nagoya Protocol, sustainable valorization of biodiversity, bioactivity of natural products, metabolomics, phytopharmaceutical technology, clinical trials and other related subjects. The manuscripts have been reviewed by the Organizing Committee members, Prof. Katsuyosi Matsunami, Prof Gunawan Indrayanto, and Prof. Angela Calderon, and edited by Dr Pawan Agrawal. The manuscripts underwent further rigorous peer review and were revised before being accepted for publication.

This special issue of Natural Product Communications is intended to help readers gain knowledge from the contributors, as well as to provide an overview of the various fields to improve natural products research.

We would like to present a special thanks to the authors and reviewers. Also, we are grateful to Dr Pawan K Agrawal, the Editor-in-Chief of Natural Product Communications and the editorial team for their assistance in the preparation of this issue and for the continued support and collaboration between Universitas Airlangga, Surabaya, Indonesia and NPC.

Prof. Bambang Prajogo EW
Chairman of the Organizing Committee
Bromo Conference,
Symposium on Natural Product and Biodiversity
Faculty of Pharmacy,
Universitas Airlangga, Surabaya
Indonesia
A lectin from the green Venezuelan marine alga *Caulerpa serrulata* was extracted with phosphate buffered saline (PBS) using cold steeping infusion (CSI) and by grinding with liquid nitrogen (GLN). The proteins were precipitated using solid ammonium sulfate. Both the crude extracts and ammonium sulfate precipitated proteins were tested for hemagglutinins using native and papain-treated human red blood cell suspensions in isotonic saline solution. Purification of lectins was achieved using affinity chromatography-sugar-epoxy-sepharose 6B and molecular weight was assessed by size exclusion chromatography using Bio-gel® P-100 and SDS-PAGE with 2-mercaptoethanol. IEF-urea 8M was also evaluated. Using CSI it was shown that the marine alga released hemagglutinating compounds into the solutions; the same hemagglutinating compounds were also obtained by GLN. Ammonium sulfate precipitated proteins exhibited agglutinating activity against native and papain-treated human red blood cells. Temperature and EDTA were shown to affect dramatically the lectin activity towards red blood cells. A lectin was purified efficiently and the molecular weight calculated as approximately 78,000 Daltons. The CSI technique demonstrated that the alga could be returned to an active metabolic state by immersion in a simple buffer after having been kept dormant by freezing at –20°C for long periods. It was also shown that the alga was releasing bioactive compounds into the solutions and, therefore, this procedure is being suggested as a good, gentle, non-disruptive extraction technique and we postulate CSI as a possible bioreactor for the continuous production of bioactive compounds from green marine alga.

**Keywords:** Lectins, Marine alga, *Caulerpa serrulata*, Cold steeping infusion, Grinding with liquid nitrogen.

Lectins were found in higher plants during the final years of the nineteenth century, and their activity first established by Stillmark [1]. Lectins were reported in marine algae for the first time over fifty years ago [2], but since then have been studied by many research groups around the globe.

Michalak and Chojnacka [3] published a review in which they presented novel methods that are used for the conversion of algal biomass into extracts with biologically active compounds, without degradation. A range of active compounds were presented, but no mention was made of lectin extraction techniques and methods. Extraction techniques to obtain lectins from marine algae mainly use either mechanical disruption in a conventional mixer/blender or freezing the alga in liquid nitrogen followed by grinding the frozen material in a mortar to a fine powder, using buffer solutions to let the compounds diffuse into the liquid [4-7].

The lectin contents of many algae do not appear to be constant, and seasonal variation in lectin content has been reported for a Japanese red alga [8]. Moreover, Hori and co-workers [8], as well as Blunden and Rogers [9] reported that the methods of preparation and storage of marine algal extracts may affect the end point values of lectin activity. In this regard, Ingram [10] suggested that such difference in lectin content might reflect either the stage of the reproductive organs or different phases of the life cycle. It was also stated that the biological function of lectins in marine algae could be a defense mechanism against potential environmental pathogen infections [10], as well as occurring in plant tissues where lectins could be responsible for an induced response to pathogenic infections and oxidative stress [11].

It has been reported that the protein content of seaweeds varies according to the species and it is generally accepted that the protein fraction of brown seaweeds is lower in comparison with that of green and red seaweeds, and the percentage of protein on a dry weight basis goes from 3 to 47% [12].

An observation made by Medina and Djabayan (data not published) revealed that a small amount of liquid residing in the plastic bags in which marine algae were transported, under cold temperature conditions, from the sea to the laboratory had hemagglutination properties. From this observation a novel technique of extraction has been developed by us, namely cold steeping infusion (CSI).

It was also reported that *Spirogyra* spp. can excrete large amounts of extracellular polymeric substances consisting mainly of polysaccharides, proteins, nucleic acids and fats [13]. The same property of excreting bioactive compounds was showed by *Ulva fasciata* when grown in a basic medium at 4°C and irradiated with a...
broad spectrum low wattage lamp. This allowed a simple in vivo study of protein biosynthesis in marine algae to be undertaken [6].

Based on all of these observations, the present work compared two different extraction techniques in which cold steeping infusion (CSI) was compared with grinding in liquid nitrogen (GLN), which is a conventional technique widely utilized by researchers in this field. Comparison of the products obtained from these procedures has been carried using preparative and analytical methods.

The most preferred extraction solutions for algal samples are isotonic saline solution, 0.85% sodium chloride (ISS) and phosphate buffered saline (PBS) [2, 14]. No comparison of extraction efficiency of solutions was found in the literature reviewed. Therefore, the marine alga Caulerpa serrulata was defrosted, cleaned of associated macro-organisms, sand and other debris, and the algal products obtained both by disruption of frozen tissue using liquid nitrogen and grinding in a mortar to a fine powder, and by CSI, in which disrupted tissue and intact alga were submerged in PBS to let the products diffuse into the buffer.

The protein concentrations of the extracts were estimated using the Bradford assay, but the standard curve for this was found to fit better as a second order polynomial than a linear relationship reported in “Protein Methods” [15]. Total soluble protein content in 100 g of wet alga following CSI and GLN was calculated as 715.6 mg (CSI gave 407.2 mg and GLN 308.4 mg). The water content was estimated to be 64.9% and, therefore, 715.6 mg of total soluble proteins represents 2.0% of the dry weight of alga.

Generally, human and animal erythrocytes are used to assess the biological activity of marine algal extracts. More sensitive results have been found with rabbit erythrocytes as compared with either human or other animal red blood cells [8]. The enhanced sensitivity observed with rabbit erythrocytes over other species has been verified by many researchers. [16-21].

Enzyme treatment of red blood cells removes glycoproteins from the external erythrocyte membrane decreasing the negative charges, as well as reducing stearic hindrance [22]. The results obtained by Ainouz and co-workers [23] showed that papain treated erythrocytes were far more efficient for detecting agglutinins than erythrocytes treated with other enzymes. Moreover, the addition of bovine albumin at a 30% concentration [2] and N-acetylglucosamine [24] increases by several folds the hemagglutination titer values of lectins.

Native and papain-treated human erythrocytes were used in the present work and were shown to be very efficient for the assessment of lectin activity of the extracted compounds from C. serrulata. The semi-quantitative estimation of hemagglutination activity was determined visually. Crude extracts and ammonium sulfate precipitated protein samples from both extraction techniques were shown to agglutinate strongly native and papain-treated human A₁, B, A₁B and O erythrocytes. The same agglutination activity was reported by Ainouz and Sampaio [21] when they studied twenty Brazilian marine algal species, among them Caulerpa cupressoides, which agglutinated human A, B, and O erythrocytes equally.

Most of the marine algal lectins studied in detail have been from the Rhophophyceae, followed by Chlorophyceae and Phaeophyceae [25]. It was suggested that most marine algal lectins are low molecular weight proteins or glycoproteins. Algal lectins have variable affinity for monosaccharides, oligosaccharides and glycoproteins and most of them require divalent cations for the integrity of their biological activity. They mainly occur as monomeric forms and have a high content of acidic amino acids, which gives them in most cases isoelectric points (pI) between 4.0 to 6.0 [26, 27].

Various sugars were assessed for their ability to inhibit the agglutination activity of ammonium sulfate precipitated C. serrulata proteins towards human papain treated A₁ blood group erythrocytes. The proteins were dissolved to a final concentration of 0.5 mg mL⁻¹. At this concentration the solution contained 4 agglutination units mL⁻¹. N-acetyl-D-galactosamine (minimum inhibitory concentration [MIC] 1.56 mM) and N-acetyl-neuraminic acid (MIC 0.78 mM) were the only two monosaccharides able to inhibit the agglutination of papain-treated erythrocytes from blood group A₁. What is more, N-acetyl-neuraminic acid showed a high level of inhibition of the hemagglutination.

The hemagglutination activity of the ammonium sulfate precipitated proteins on a 5% suspension of human A₁ erythrocytes treated with papain was affected by heating; the activity decreased substantially at 50°C and was absent from 60°C onwards.

Addition to the PBS buffer of 10 mM EDTA completely abolished the agglutination activity of the ammonium sulfate precipitated proteins against human papain-treated A₁ blood group erythrocytes. Therefore, the binding reaction between the sugar moieties and the protein is cation dependent. Recovery of the activity was achieved by the addition to the mixture separately of either a solution containing 100 mM of CaCl₂ or a solution of 100 mM MnCl₂.

The isolation of lectins from marine algal extracts has generally employed affinity chromatography and uses an insoluble carrier to which a specific carbohydrate that strongly binds to the lectin under investigation is attached. Separate 2 mL solutions of precipitated proteins obtained using both CSI and GLN as extraction techniques were applied to a GalNAc-sepharose 6B column and hemagglutination activity of the fractions was assessed using papain-treated human A₁ group erythrocytes. The obtained profiles were very similar to each other. That obtained for the CSI proteins is shown in Figure 1.

Active fractions were pooled and dialyzed extensively against distilled water and lyophilized. The amount of protein bound to the columns was estimated; CSI produced 4.2 mg and GLN 4.7 mg (8.9 mg of lectin in total). Both products obtained strongly agglutinated papain-treated blood group A₁ human erythrocytes.

SDS-PAGE with 2-mercaptoethanol showed that proteins obtained by affinity chromatography from C. serrulata were not stained using Schiff’s reagent, and two similar bands of high molecular weight of probable polysaccharide contaminants were observed in tracks 1 and 2. Meanwhile, two very similar bands were obtained in the running tracks 1 and 2 with almost identical molecular weight: 20,000 Daltons (Figure 2).

Protein molecular weight was calculated using size exclusion chromatography bio-gel® p-100. The lyophilized proteins precipitated with ammonium sulfate obtained by CSI and GLN were dissolved in degassed PBS, pH 7.3, containing 0.01% sodium azide to a final concentration of 10 mg mL⁻¹. Two mL was applied to the column and the activity of each fraction was assessed using hemagglutination tests against papain treated blood group A₁ human erythrocytes. The protein profile of both the CSI and GLN extracts were very similar. Three peaks (P₁, P₂, and P₃) were observed in each. The profile of the CSI extract is shown in Figure 3. Molecular weight (Mr) was calculated in both profiles as: P₁ = 77,600 Daltons, P₂ = 42,650 Daltons, and P₃ = 6,200 Daltons. Fractions 16 to
In this paper we report a novel technique to extract lectins from marine algae that we named cold steeping infusion (CSI), which was compared with extraction by grinding with liquid nitrogen (GLN). The CSI procedure demonstrated that the alga could be kept dormant by freezing at -20°C for long periods. It was also shown that the alga was releasing bioactive compounds into the solutions. Our results showed that the same proteins (lectins) were extracted from *C. serrulata* using both extraction techniques and the lectins exhibited the same biological activities and had the same molecular weight. Therefore, this procedure is suggested as a good, gentle, non-disruptive extraction technique and we postulate CSI as a possible bioreactor for continuous production of bioactive compounds from green marine algae.

**Experimental**

**Source of alga:** The green Venezuelan marine alga, *Caulerpa serrulata* Forsskål (family Caulerpaceae) was collected from Los Juanes (Morrocoy National Park) Tucacas, Falcon State, Venezuela. The alga was frozen and transported from the sea to the laboratory in cold boxes containing dry ice. It was defrosted as required, and cleaned of associated organisms, sand and other debris using distilled water prior to any further experimentation. A voucher sample is lodged in the Herbarium of the Facultad de Farmacia y Bioanálisis, Universidad de Los Andes, Mérida, Venezuela.

**Buffer and solutions preparation**

(i) **Buffer:** Phosphate buffered saline (PBS) 0.153M, pH 7.3, was prepared by dissolving 1.878 g of NaH₂PO₄ plus 8.5 g of Na₂HPO₄ 4.5 g of NaCl in distilled water and made up to 1 L. pH was checked using a pH meter.

(ii) **Solution:** Isotonic saline solution (ISS) 0.145M, pH 7.3 (0.85%), was prepared by dissolving 8.5 g of NaCl in distilled water and the final volume made up to 1 L. pH was checked using a pH meter.

**Protein extraction**

(i) **Cold steeping infusion (CSI):** Marine alga (50 g) was submerged in 100 mL of PBS, 0.153M, pH 7.3 and incubated at 4°C for 48 h under a permanent artificial source of light. The resulting solution was collected and the volume readjusted to 100 mL and stored at -20°C for further investigations.

(ii) **Grinding with liquid nitrogen (GLN):** The alga (50 g) was placed in a stainless steel bowl and covered with liquid nitrogen. Using a pestle and mortar the frozen alga was ground to a fine powder, which was added to 100 mL of PBS, pH 7.3, and incubated at 4°C for 48 h. Particulate matter was removed by initially straining through a household strainer, 1 mm mesh, followed by centrifugation at 5000 rpm (MSE, Centaur 2 Centrifuge) for 10 min. The supernatant was collected and the volume readjusted to 100 mL and stored at -20°C for further investigations.

**Proteins salting out:** The crude extracts obtained were treated at 4°C with 56.8 g of solid ammonium sulfate to achieve 85% sulfate saturation. After the salt had dissolved, the mixture was left gently stirring for 60 min. The precipitates produced were collected by centrifugation at 10,000 rpm (Jouan MR-1812 Refrigerated Centrifuge, maintained at 4°C) for 15 min, and then dissolved in the minimum quantity of PBS. The resulting solution was again centrifuged, using the previous conditions, the supernatant exhaustively dialysed against distilled water, lyophilised and stored at -20°C until required [28].

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**Figure 1:** Affinity chromatographic profile using GalNAc-sepharose 6B column of *Caulerpa serrulata* precipitated proteins using ammonium sulfate. Technique of extraction: CSI. Buffer: Degassed PBS, pH 7.3. Hemagglutinating activity of fractions determined using papain-treated human blood group A, erythrocytes. The arrow indicates the application point of degassed 1M NaCl.

**Figure 2:** SDS-PAGE with 2-mercaptoethanol of proteins from *Caulerpa serrulata* obtained by CSI and GLN and purified using GalNAc-sepharose affinity chromatography column. A, 13% acrylamide gel stained using Schiff’s reagent, B, 13% acrylamide gel stained using Coomassie® Brilliant Blue R. Track 1 = CSI and Track 2 = GLN. Track 3 proteins of known molecular mass used as markers.

**Figure 3:** Size exclusion chromatography profile of *Caulerpa serrulata* precipitated proteins using ammonium sulfate. Method of extraction: CSI. Buffer: Degassed PBS, pH 7.3. Hemagglutinating activity of fractions using papain-treated human erythrocytes from blood group A₁.
**Protein concentration:** The protein concentration of the marine algal extract was estimated using the Bradford assay (Coomassie Brilliant Blue-R, phosphoric acid and methanol) (Sigma Product No.B-6916) and bovine serum albumin, 2 mg mL\(^{-1}\) as standard (Sigma Product No. P-0834). Protein standard solutions of 5, 10, 15, 20, 25, 30, 35 and 40 μg mL\(^{-1}\) were prepared by diluting the appropriate amount of bovine serum albumin in PBS. Absorbance values were measured at 595 nm (\(A_{595}\)) using a Perkin-Elmer 628-lambda 5-3 UV/vis spectrophotometer employing quartz cuvettes with 10 mm optical path length. The standard curve for the Bradford Assay reported in “Protein Methods” [15] shows a linear relationship. A more detailed analysis of the data reported and presented by them in their book, showed that a second order polynomial with a correlation coefficient of 0.999 was a better fit than the published linear relationship with a correlation coefficient of 0.978.

**Assessment of hemagglutination:** Hemagglutination tests were performed by mixing 0.1 mL of the appropriate algal protein product in isotonic saline solution (ISS), 0.145M, pH 7.3 (0.85%) with 0.1 mL of each erythrocyte suspension. After gentle agitation the mixtures were left to incubate at room temperature for 30 min and the degree of agglutination assessed macroscopically on a scale of 4+, for very strong, to zero for total absence.

**Inhibition of haemagglutination activity:** Two-fold serial dilutions of each potential inhibitory monosaccharide (100 mM) or protein (fetuin 5 mg mL\(^{-1}\)) were prepared to a final volume of 0.1 mL in each tube. Ammonium sulfate precipitated proteins (0.1 mL), obtained from the alga by CSI and GLN, prepared at 4 hemagglutinating units mL\(^{-1}\) were added to each tube. These tubes were left at room temperature for 1 h before 0.1 mL of papain-treated blood group A\(_1\) erythrocytes at 5% was added to each tube and allowed to stand at room temperature for a further 1 h, followed by centrifugation for 2 min at 4,000 rpm (MSE Centaur 2 Centrifuge). The hemagglutinating inhibition titer was recorded as the highest dilution of sugar able to inhibit erythrocyte agglutination that could be produced by a hemagglutinin at 4 hemagglutination units mL\(^{-1}\).

**Effect of temperature on hemagglutination activity:** The ammonium sulfate precipitated proteins showing hemagglutination properties were dissolved in 0.85% NaCl + 10 mM CaCl\(_2\) and 10 mM MnCl\(_2\) solution, pH 7.0, to a final concentration of 5 mg mL\(^{-1}\). Each algal solution (1 mL) was heated separately in a water bath (Bird and Tatlock London Limited, Chadwell Heath Essex, England) for 30 min at the following temperatures (40, 50, 60, 70, 80 and 90°C). Hemagglutination activity was tested before and after the heating procedure using a 5% suspension of human A\(_1\) erythrocytes previously treated with papain.

**Effect of EDTA on hemagglutination activity:** The procedure described for assessing agglutination was modified by changing the ISS solution to a 0.85% NaCl solution containing 50 mM EDTA. This was used to dissolve the ammonium sulfate precipitated protein and to prepare the 5% papain-treated human blood group A\(_1\) erythrocyte suspension. The dissolved proteins were mixed in turn with equal amounts of erythrocyte suspensions and the mixture left to incubate at room temperature for 1 h. The hemagglutination activity was then assessed. The recovery of any observed loss in agglutination activity was tested by adding equal amounts of 0.85% NaCl solution, containing 100 mM CaCl\(_2\) and 100 mM MnCl\(_2\) to the appropriate EDTA containing solutions.

**Isolation of lectins:** Ammonium sulfate precipitated proteins, obtained from the alga by CSI and GLN in PBS, pH 7.3, containing 0.01% of sodium azide, were processed further using affinity gel chromatography. A Sepharose 6B–GalNac complex was prepared, essentially as described by Vreblad [29], but with the temperature used for coupling the sugar to the Sepharose reduced from 45° to 30°C.

The resulting gel was packed into a 10.5 x 0.8 cm column and equilibrated with 0.85% NaCl, 10 mM CaCl\(_2\), pH 7.0, solution. Protein (10 mg), dissolved in 2 mL of the same solution, was added to the column which was developed until all unbound material had been eluted. The eluting solution was changed to 0.85% NaCl, pH 7.0: 1 mL fractions were collected, and their absorbance values at 280 nm measured together with their hemagglutinating activity. Where appropriate, fractions were pooled together, dialysed extensively against distilled water and lyophilised.

**Molecular weight (Mr) estimation:** The Mr of the extracted lectin was assessed by size exclusion chromatography using Bio-gel\(^{P}\) P-100. Lyophilized proteins precipitated with ammonium sulfate obtained by CSI and GLN were dissolved with degassed PBS, pH 7.3, containing 0.01% of sodium azide to a final concentration of 10 mg mL\(^{-1}\) and then applied to a packed and equilibrated gel into a glass column (Pharmacia) 70 x 1.5 cm (total volume of ~124 cm\(^3\)), using degassed PBS at a flow rate of 12 mL h\(^{-1}\) at 4°C; 2mL fractions were collected. The standard curve was obtained from individual protein standards as a second order polynomial curve with a correlation coefficient of 1.000.

**Electrophoresis:** Electrophoresis (SDS-PAGE) was carried out essentially as that described by Laemmli [30] using a constant voltage of 200V and bromophenol blue as a migration marker.

(i) **Staining to reveal the presence of carbohydrate:** Following electrophoresis, gels were fixed with trichloroacetic acid (12.5%) and immersed in a 1:1 mixture of 1% periodic acid and 3% acetic acid for 50 min in order to oxidise any sugar moieties. Following washing with distilled water, gels were immersed in Schiff’s fuchsin-sulfite reagent (Sigma Aldrich Co., Ltd., UK) and incubated in the dark for 50 min. The latter was replaced by 0.5% (w/v) sodium metabisulphite solution for 10 min. The final step was repeated 3 times; any background staining was removed by washing the gels in distilled water overnight using a shaker.

(ii) **Protein staining:** A second staining procedure was carried out by immersing the Schiff’s reagent stained gels in Coomassie Brilliant Blue-R stain solution for 15 min and destaining with a mixture of 10% acetic acid and 25% methanol.

**Isoelectric focusing (IEF) of proteins:** Isoelectric focusing was carried out as described by Righetti [31] and Allen et al. [32]. Protein standard solution and ammonium sulfate precipitated proteins obtained from the alga by CSI and GLN were applied to polyacrylamide-ampolyphile gel plates, which were initially developed using a constant voltage setting of 150V for 30 min followed by 200V for a further 2.5 h and bromophenol blue as a migration marker. The pH gradient was determined and gels were fixed with 10% trichloroacetic (TCA) for 10 min and replaced by 1% TCA overnight. The same stain solution and procedure for the protein electrophoresis gels were used.

**Acknowledgments** - We wish to acknowledge the University of Portsmouth, United Kingdom, University of Los Andes, Venezuela and University Nacional of Chimborazo, Ecuador.
A novel lectin extraction technique

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**Bambang Prajogo**

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