Antioxidant activity of bitter leaves (*Vernonia amygdalina*) extract coated with the nanochitosan derived from parrot fish (*Scarus* sp) scales

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Abstract *Vernonia amygdalina* is traditionally used in various folk medicines as remedies against many diseases due to its antioxidant compounds. The ability of these compounds could be reduced during absorption process. Chitosan nanoparticles as drug carriers have the advantages of protecting bioactive compounds of the extract. Nanochitosan was prepared from the parrot fish (*Scarus* sp) scales, using ionic gelation method. Extraction of bitter leaves was performed by maceration method using 96% ethanol. The nanochitosan coated extracts were prepared into 2 groups, with the ratio of nanochitosan and extract of 1:1 (BLENC 1) and 2:1 (BLENC 2), respectively. The antioxidant activity of the two groups with different concentrations (10 to 50 μg/ml) and the control (BLE) was measured using DPPH test for the parameter of inhibition values. Results show that the BLEN C 2 with concentration of 50 μg/ml has higher inhibition value (89.1%) than the 50 μg/ml concentration of BLE and BLEN C 1. The inhibition value of all samples were in range of very strong antioxidant category with the IC₅₀ ranged from 8.98 to 22.25 μg/ml. Linear regression of the concentration against inhibition value graph shows that correlation was found to be in BLEN C 2 ($R^2=0.828$).

Keywords : *V. amygdalina*, antioxidant, nanochitosan, inhibition value

1. Introduction
Pharmaceutical interests for medicinal plants are mostly due to their antioxidant and free radical scavenging properties. Free radicals had been reported to be responsible for the pathophysiological of several diseases such as cancer, diabetes, and cardiovascular diseases [1] Antioxidant has a capacity to dissolve free radicals of cells in human bodies [2]. One of those medical plants is bitter leaf (*Vernonia amygdalina*) which belongs to the Compositae family. The name bitter leaf originated from the bitter taste of leaves and stem. Previous studies have reported that bitter leaves have antimicrobial, antidiabetic, antimalarial, anticancer, antioxidative and hypolipidaemic effects [3,4]. These compounds may be unstable in gastro intestinal tracts due to elevated pH and harsh conditions that render the biotherapeutic compound ineffective in gastrointenstinal tracts.

One of the promising ways to prevent these problems is coating the extracts with nanoparticles. Applying nanotechnology to plant extracts has revealed an advantageous strategy for herbal drugs considering the numerous features of nanostructured systems [5]. Nanoparticles including nanochitosan...
are potentially used in drug delivery system, due to its versatility in targeting tissues and enabling deep molecular targets [6]. Chitosan is an N-acetylated derivative of chitin widely used as a building material for nanoparticles [7]. It has excellent biodegradable, biocompatible, nontoxic and nonimmunogenic properties. It prolongs drug release time in the gastrointestinal tract. A new source of chitosan can be obtained from marine fish scales as reported by [8].

Fish scales are abundantly available as wastes of fish processing in Manado, North Sulawesi which popularly known as a city surrounding with seafood restaurants. Parrot fish is one of the preferred marine fish species in various restaurants, and this fish was sold after removing the scales in local markets. Modifying the particle size to nanoparticles can enhance its ability as a coating agent. Many studies have reported that plant extract coated by nanochitosan can improve the efficacy of result in expression of bioactivity of plant extract [9]. However, until now, there is no report on the antioxidant properties of \textit{V. amygdalina} extract coated with the marine derived nanochitosan. This study is part of an ongoing research aimed to determine the proper dose of nanochitosan as a coating agent of the extract of bitter leaves for optimizing its antioxidant capacity.

2. Material and Methods

2.1. Plant extraction
The bitter leaves extraction were prepared according to the method described by [10] with slight modifications. Bitter leaves were sorted and washed with running tap water. Leaves were dried at room temperature, smoothed using a blender and filtered through 177 µm mesh screen. The functional compounds of bitter leaves powder were extracted by maceration method using 96% ethanol. Approximately 217 g bitter leaves powder was macerated in 2000 ml of 96% ethanol for 72 hours. The filtrate was evaporated using a vacuum evaporator then heated in an oven at 34°C for 24 hours.

2.2. Extraction of chitosan and modified to nanochitosan
The parrot fish scales (\textit{Scarus} sp) were collected from local markets in North Sulawesi. The fish scales were washed and solar-dried for two days. The procedure of chitosan extraction was adopted from [11] with modification. The pre-treatment step was carried out using NaOH 0.5 M solution for 48 hours, followed by hydrolysis with HCl 0.75 M solution for 24 hours. The solution was then neutralized with distilled water, and heated at 110°C for 1 hours with NaOH 40% and repeatedly neutralized. The chitosan was obtained by separating residue from solution. Preparation of nanochitosan using the ionic glass method, by adding 0.1% Tween 80 as a homogenizer and 0.1% tripoliposphate (TPP) as a stabilizer. This procedure of nanochitosan preparation from the chitosan modified from the fish scale derived chitin has been submitted for an Indonesian patent with registered number of P14201802743 April 13, 2018

2.3. Coating bitter leaves extract with nanochitosan
Coating of bitter leaves extract with nanochitosan refers to [12] method with modification. The coating process is carried out by adding nanochitosan into the extracts with ratio of 1 : 1 (BLENC 1) and 2:1 (BLENC 2), respectively, and followed by homogenizing and centrifugating with 2000 rpm for 10 minutes. Besides these two treatment, there was a control, the extract without coating (BLE).

2.4. Determination of extract yield (%) and phytochemical screening
The percentage yield of extract and chitosan was obtained by dividing the initial weight and the final extract weight then multiplying the ratio by 100. The phytochemical analysis was performed for identification of the functional target compounds, such as alkaloids, flavonoids, tannins and terpenoid [13].
2.5. DPPH radical scavenging activity
The ability of *V. amygdalina* leaves extracts to scavenge stable DPPH radical was measured using the method of [14]. Five different concentrates ranged from 10 – 50 µg/ml of each treatment (BLE, BLENC1 and BLENC2) in methanol were prepared in test tubes, so there were 15 tubes (Tabel 2). One milliliter of 0.3 mM of freshly prepared DPPH solution in methanol was added to 2.5 ml solution in each tube and allowed to react in the dark at room temperature for 30 min. Absorbance of each solution was measured at 518 nm. As a blank, 1 ml of methanol was added to 2.5 ml of each extract solution with no DPPH. As negative control, 1 mL of 0.3 mM DPPH solution added to 2.5 mL of methanol. Percentage DPPH inhibition activities of the extracts and standards were determined using the equation

Scavenging activity (%) = \[ \left( \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \right) \times 100 \]

where \( \text{Abs}_{\text{control}} \) is the absorbance of DPPH radical + methanol, and \( \text{Abs}_{\text{sample}} \) is the absorbance of DPPH radical + sample extract/standard. The results were expressed as IC\(_{50}\), which means the concentration at which DPPH radical was quenched by 50%. The concentration of extract providing 50% inhibition (IC\(_{50}\)) was calculated from the plotted graph of % inhibition versus concentration curve.

3. Results and Discussion
3.1. The percentages of chitosan yield
The yield percentage from fish scales to chitin was 50% and chitin to chitosan was found to be 14.2%. The amount of 800 g dry fish scales were processed and yielded 400 g chitin and further resulted in 56.8 g chitosan. The characterization of chitosan and nanochitosan from fish scales with particle size ranged from 56-291 nm, have previously been reported [15].

3.2 The percentages of and phytochemical screening of bitter leaves extract
The yield of ethanol extract obtained by maceration of bitter leave extract (BLE) was about 9.22%. The results obtained from the process of extraction was about similar to that of a previous study [16]. Phytochemical screening results showed that functional compounds such as alkaloid, flavonoids, saponin, and triterpenoid were detected, while tannins was absent (Table 1).

| Compound   | Result |
|------------|--------|
| Alkaloid   | +      |
| Flavonoids | +      |
| Tannins    | -      |
| Saponin    | +      |
| Triterpenoid| +      |

+ : detected - : not detected

The results of phytochemical screening are similar with a study of [17] who reported that tannins were not present in the ethanolic extract of bitter leaves, only the alkaloids, steroids and flavonoids were present. These compounds are secondary metabolites with a definite physiologic action on the human body [18,19], may act in synergy with other existing phytochemicals in the bitter leaves extracts to produce the medicinal benefits.

3.3. Antioxidant activity results
Antioxidant activity of the 3 samples was manifested through their percentage inhibition of DPPH radical. BLE; BLENC 1; and BLENC 2 exhibited appreciable scavenging activity ranging from 81.59% to 88.976% (Figure 1); 64.079% to 87.664% (Figure 2) and 50.844% to 89.126% (Figure 3) for concentrate extract 10-50 µg/ml, respectively. In the other hand, the bitter leaves extract coated by nanochitosan with ratio 2:1 (BLENC2) showed the higher inhibition value of 89.126% at 50 µg/ml while BLE and BLENC 1 were found to be 88.976% and 87.664% respectively at the same concentrate extract,
as shown in Table 2. Although the value did not show a significant difference, nanochitosan proving its ability as a coating to maintain the stability of antioxidant at the right concentrate.

Table 2. Antioxidant activity by DPPH radical scavenging activities

| Absorbance of control | Treatment | Concentrate (μg/ml) | Bitter leaves Extract | Absorbance | Data 1 | Data 2 | Data 3 | Average | % Inhibition |
|-----------------------|-----------|--------------------|-----------------------|------------|-------|-------|-------|--------|--------------|
| 0.889 | BLE | 10 | 0.163 | 0.165 | 0.163 | 0.164 | 81.590±0.129 |
| 20 | 0.14 | 0.156 | 0.146 | 0.147 | 83.427±0.909 |
| 30 | 0.12 | 0.122 | 0.126 | 0.123 | 86.202±0.343 |
| 40 | 0.103 | 0.107 | 0.101 | 0.104 | 88.339±0.343 |
| 50 | 0.098 | 0.086 | 0.09 | 0.092 | 88.976±0.674 |
| 0.889 | BLENC1 | 10 | 0.326 | 0.31 | 0.322 | 0.319 | 64.079±0.936 |
| 20 | 0.298 | 0.259 | 0.256 | 0.271 | 69.516±2.635 |
| 30 | 0.211 | 0.209 | 0.21 | 0.21 | 76.378±0.112 |
| 40 | 0.166 | 0.164 | 0.164 | 0.165 | 81.477±0.129 |
| 50 | 0.109 | 0.11 | 0.11 | 0.11 | 87.664±0.064 |
| 0.889 | BLENC2 | 10 | 0.471 | 0.436 | 0.404 | 0.44 | 50.844±3.769 |
| 20 | 0.34 | 0.389 | 0.401 | 0.38 | 57.705±3.762 |
| 30 | 0.305 | 0.302 | 0.3 | 0.3 | 65.992±2.838 |
| 40 | 0.293 | 0.29 | 0.291 | 0.29 | 67.229±0.171 |
| 50 | 0.10 | 0.09 | 0.1 | 0.1 | 89.126±0.005* |

Values are mean ± SD of three (3) results; * show the higher inhibition values compared to the BLE.

Similar findings have been observed by [20] who reported that Egyptian prickly pears peels fruit coated by nanochitosan showed an increase in the inhibition value. The present findings have proved the efficacy of nanochitosan as a coating agent, as in our previous study on phenolic compound of basil leaves extract [21]. Coating with nanochitosan could inhibit the growth of calcium oxalate crystals of *Tridax procumbens* L [22].

The Value of free radical damping activity is expressed by IC₅₀ (Inhibitory Concentrate fifty). If the IC₅₀ value of an extract is below 50 μg/ml then the antioxidant activity is in very strong category. The antioxidant activity in the strong category if the IC₅₀ is in the range of 50-100 μg/ml, while range of 100-150 μg/ml means the antioxidant activity is in moderate category and 150-200 μg/ml means that antioxidant activity is in weak category. Whereas the IC₅₀ value is in very weak category if the value under 200 μg/ml [23].

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**Figure 1.** DPPH radical scavenging activity of Bitter leaves extract (BLE)

**Figure 2.** DPPH radical scavenging activities of bitter leaves extract coated by nanochitosan 1:1 (BLENC1:1)
Each value is expressed as mean ± SD (n=3)
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