Nutritional Composition and Topoisomerase Inhibitor Activity of Ethnomedicinal Marine Mollusk *Nerita albicilla*

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Abstract: DNA topoisomerases (topo) I and II are molecular targets of several potent anticancer agents. Thus, inhibitors of these enzymes are potential candidates for anticancer development. Traditionally, *Nerita albicilla* had been used in Kei Island, Southern Maluku, Indonesia to treat liver disease including cancer. The paper reports on the chemical composition of *Nerita albicilla* and its topo I inhibitor of hexane, ethyl acetate and methanol extracts. Topoisomerase-I inhibitor activity was determined using the method reported by TopoGEN. The proximate analysis described that *Nerita albicilla* dried powder contained 12.45% $\pm$ 0.05% moisture; 9.17% $\pm$ 0.03% ash; 62.05% $\pm$ 0.10% protein; 5.58% $\pm$ 0.08% fat; 6.60% $\pm$ 0.02% crude fiber and 4.15% $\pm$ 0.24% carbohydrate (by difference). Furthermore, the protein consisted of 11 essential amino acids and six non-essential amino acids. It contained significant amount of branched-chain amino acids (BCAA) valine, leucine, isoleucine (a total of 187.8 mg g⁻¹ protein) and lower content of aromatic amino acids phenylalanine, tyrosine and histidine (a total of 111.26 mg g⁻¹ protein). The protein score was 92.2. The yield of hexane, ethyl acetate and methanol extracts of *Nerita albicilla* were 2.05% $\pm$ 0.05%, 1.56% $\pm$ 0.06% and 6.99% $\pm$ 0.14%, respectively. All extracts showed topoisomerase-I inhibitor activities. Minimum inhibitory concentration (MIC) of methanol extract was 2.50 μ g mL⁻¹. Chemical screening of the extracts showed that they contained steroidal and alkaloid compounds. The investigation revealed that *Nerita albicilla* contains active compounds that could be potential for nutraceutical or pharmaceutical development.

Key words: *Nerita albicilla*, nutritional composition, topoisomerase-I inhibitor.

1. Introduction

Nerita albicilla named locally as Kablang is a marine mollusk used traditionally to cure liver disease including cancer in Kei island, Tual, Southern Maluku, Indonesia. The taxonomic of Kablang was provided by National Institute of Oceanology, the Indonesian Institute of Science (LON-LIPI). Traditionally, Kablang was prepared by boiling in water and the water and flesh taken orally. *Nerita albicilla* contained oxindol alkaloid named isopteropodine [1]. Isopteropodine is also found in roots and in smaller quantities in leaves and bark of *Uncaria tomentosa*, the

member of Rubiaceae family. In Western Europe, *Uncaria tomentosa* has been used as folk medicine possessing immunostimulating properties [2], and extracts positively modulate the function of rat muscarinic M1 and 5-HT₂ receptors expressed in *Xenopus oocyte* [3]. Isopteropodine (uncarines E) also exhibited weak but consistent cytotoxic effect on mouse fibroblasts and tumor cell lines human (non-microcellular lung cell carcinoma, cervical carcinoma, prostate carcinoma) and mouse (reticular lymphosarcoma, stomach carcinoma) with the mechanism of inhibiting topoisomerase-I [4]. The commercially available extracts used in Western Europe (e.g., Krallendorn[®] manufactured by Immodal

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Pharmaka GmbH) are the most frequently standardized so as to obtain oxindole alkaloid concentration of 1.3%-1.75%, with 97% of their content accounting for pentacyclic alkaloids (Falkiewicz&Tukasiak, 2001). Krallendorn is a prescription drug registered as an adjunctive therapy for rheumatoid arthritis [5].

Nerita albicilla also contained an antibacterial pigment named fulvoplumierin [6]. It was also found in Indonesian plant *Plumeria rubra* and reported as cytotoxic against murine lymphocytic leukemia (P-388), human cancer cell type (breast, colon, fibrosarcoma, lung, melanoma, KB) [7]. Fulvoplumierin is an inhibitor of HIV-1 type reverse transcriptase [8].

The suspected target of anticancer isopteropodine is the topoisomerase enzyme. This is an essential nuclear enzyme that plays a vital role in a variety of cellular processes by maintaining the superhelical density of DNA. It consists of topoisomerase-I which catalyzes the relaxation of supercoiled DNA and topoisomerase-II that introduces double-stranded breaks. Topoisomerase-I inhibitors stabilize the transient enzyme-DNA complexes, resulting in an inhibition of transcription and replication that ultimately leads to DNA damage and cell death. Topoisomerase is found excessively in cancer cells compared to normal cells. For this reason, topoisomerase inhibitor has been used as target molecule for anticancer development by drug companies [9, 10]. Two derivatives of camptothecin, topotecan and irinotecan have successfully entered into the market and are used as topoisomerase-I poisons in clinical practice [11]

The choice of ethnomedicinal organisms provide several advantages including reducing problems relating to toxicity as the organisms have been used traditionally for decades, ethnically, it has been proven to treat various diseases so the chance to pass the efficacy test is higher. Previous study revealed that *Nerita albicilla* contained valuable bioactive

compounds such as isopteropodine and fulvoplumierin. However, there is no report on the nutritional value of *Nerita albicilla*.

This research was conducted to support its traditional usage and to explore its possible use for nutraceutical development, thus the aims were to investigate the nutritional compound of *Nerita albicilla* and its activity as topoisomerase-I inhibitor.

2. Materials and Methods

2.1 *Nerita Albicilla* and Preparation of Extracts

About 350 g (90-100 pieces) of *Nerita albicilla* was collected from sandy beach area at Kei Island, Tual, Southern Maluku. The identification was carried out at National Institute of Oceanology, the Indonesian Institute of Science (LON-LIPI), Jakarta. The flesh was removed from the shell, washed, sun dried and powdered. The finely ground sample of 20 g was extracted sequentially in 80 mL using hexane, ethyl acetate and methanol. The extraction was carried out using a shaker overnight at room temperature. The crude extracts were filtered using filter paper and dried under vacuum condition at 40 °C. The extracts were kept in the dark at ± 4 °C until tested.

2.2 Nutritional Analysis

Nutritional analysis was conducted to quantify moisture, ash, crude protein, fat, carbohydrate and crude fiber content according to standard method of Association of Official Analytical Chemist [12]. Amino acids composition was determined using High Performance Liquid Chromatography (HPLC).

A sample of 0.1 g dried powder was hydrolyzed using 10 mL HCl 6 N at 100 °C for 24 hours. The hydrolyzed protein was filtered. 10 μ L aliquot was reacted with 30 μ L drying solution consisting of methanol, natrium asetat and triethylamine (2:2:1). The solution was vacuum dried and reacted with 30 μ L mixture of methanol, pycoiodothiocyante, and trimethylamine (3:3:4). After reaction, the mixture was diluted by 10 mL solution consisting of 1 M natrium

acetate in 60% acetonitril. Diluted solution was refiltered applying 0.45 μm Millipore filter. 20 μL filtered solution was injected to HPLC. The concentration of amino acids was determined using standard amino acids. The column was Waters Pico tag 3.9 $\times$ 150 nm column. The column temperature was 38 $^{\circ}\text{C}$. The eluent was a mixture of acetonitrile and Natrium asetate 1 M (6:4). The flow rate was 1 mL min^{-1} . The detector was UV 254 nm (AOAC 2000). The nutritional analysis was done triplicate.

To analyze the quality of *Nerita albicilla* protein, the essential amino acids were compared to the standard recommended by FAO/WHO. The amino acid score (AAS) was determined by 100 if the value was greater than the standard FAO/WHO. If the value was less than the standard, the AAS was calculated by dividing its concentration in the sample to its standard concentration [13, 14].

2.3 Topoisomerase-I Inhibitor Assay

Topoisomerase-I and all chemicals required for its inhibitor assay were provided as Topo I Drug Screening Kit and purchased from TopoGEN, USA. This kit included topoisomerase-I, supercoiled DNA, relax DNA, TGS buffer, camptothecin. Electrophoresis grade agarose, Sodium Dodecyl Sulfate (SDS), proteinase K and other chemicals were purchased from Sigma. Topoisomerase-I inhibitor assay was conducted as described by TopoGEN. The method allows the detection of the compound that either stabilizes the complex DNA/enzyme or otherwise inhibit catalytic activity of topoisomerase-I. The positive control of inhibitor is camptothecin, which stabilizes the cleaved intermediate complex. The standard topoisomerase-I inhibitor assay mixture was 20 μL . It contained 10 $\times$ TGS (assay/cleavage) buffer (100 $\mu\text{L mL}^{-1}$), supercoiled DNA (7.5 $\mu\text{g mL}^{-1}$), topoisomerase-I 200 units mL^{-1} . All extracts (hexane, ethyl acetate and methanol) were applied at concentration of 50 $\mu\text{g mL}^{-1}$ while camptothecin was applied at 34.84 $\mu\text{g mL}^{-1}$. The methanol extract was chosen to determine the minimum inhibitory concentration (MIC) due to its

highest yield. The selected concentration was 100.0 $\mu\text{g mL}^{-1}$, 50.0 $\mu\text{g mL}^{-1}$, 25.0 $\mu\text{g mL}^{-1}$, 12.5 $\mu\text{g mL}^{-1}$, 5.0 $\mu\text{g mL}^{-1}$, 2.5 $\mu\text{g mL}^{-1}$, and 1.25 $\mu\text{g mL}^{-1}$. The solvent used to dissolve extract or camptothecin was 10% dimethylsulfoxide (DMSO). Reaction was carried out at 37 $^{\circ}\text{C}$ for 30 min and then terminated by adding SDS 1% (100 $\mu\text{L mL}^{-1}$). Furthermore, the mixture was added with proteinase K (50 $\mu\text{g mL}^{-1}$) and incubated for 30 min at 37 $^{\circ}\text{C}$. The samples were electrophoresed in a horizontal 1% agarose gel in Tris-acetate/EDTA buffer. The gel was stained with ethidium bromide and destained in water and photographed under UV illumination. The experiments were carried out in triplicate.

The activity of topoisomerase-I is determined by the formation of relax DNA. The topoisomerase-I inhibitor has two mechanisms that are catalytic and poison. The first is observed if the substrate supercoiled DNA remains unchanged after reaction, while the second is indicated by the formation of complex DNA/Enzyme containing open circular DNA.

2.4 Chemical Group Determination of Inhibitor Topoisomerase-I

The active extract showing inhibitor topoisomerase-I was tested qualitatively according to standard method, if the extract contained protein, free amino acids, carbohydrate, steroid, triterpenoid, alkaloid, flavonoid, saponin. One gram of sample extract was dissolved in 5 mL hexane, ethyl acetate and methanol separately. Free amino acids was determined using ninhydrin reagent. One milliliter of solvent containing extract was reacted with 1 mL ninhydrin reagent, then boiled for 5 min. The presence of free amino acids other than proline and hydroxyproline resulted in violet color. Otherwise it turned to yellow.

Carbohydrate content was tested using Molisch reagent containing 5% of alpha-naphtol and 95% of ethanol. One milliliter extract was added by two drops of Molisch reagent, then added by 1 mL sulphuric acid slowly and gently. The formation of violet color

indicates that the extract contained carbohydrate. Otherwise, the mixture shows green color.

Steroidal compound was determined using Lieberman-Burchard reagent. One milliliter of extract was added by 1 mL chloroform, 10 drops of unhydrate acetic acid and two drops of sulphuric acid, sequentially. The solution was mixtured gently and left for several minutes. The formation of green-blue color indicated that it contained steroidal compound, while formation of red-violet indicated it contained triterpenoid.

Protein content was assayed using Bradford reagent, 0.1 mL extract was added by 1 mL Bradford and mixed thoroughly. The presence of protein turned the mixture into blue color. The alkaloid compound was tested by reacting extract with Dragendorff, Meyer and Wagner reagent. One gram of extract was dissolved in 10 mL chloroform and added by several drops of NH_4OH and then filtered and kept in closed tube. Furthermore, the mixture was added by 10 drops of H_2SO_4 (2 M). The acid phase (upper layer) was seperated and dropped in spot plate. Afterward the spot was reacted individually with Dragendorff (KBiI_4), Meyer K_2 (HgI_4) and Wagner KI (I_2) reagents. The presence of alkaloid resulted in red-orange, white and brown, respectively.

The saponin content was done by dissolving 1 g of extract in water and boiled for 5 min. The solution was cooled and mixed. The foam formation that stayed for 10 min indicated that it contained soaponin. Flavonoid and hydroquinone were determined by dissolving 1 g of sample in 30% hot methanol. Furthermore, the solution was filtered and the filtrate was dropped in spot plate and added drop of NaOH 10% (w/v) or H_2SO_4 separately. Formation of red color in the presence of H_2SO_4 and NaOH indicated it contained flavonoid and phenolic hydroquinone, respectively.

3. Results and Discussion

3.1 Nutritional Composition of *Nerita albicilla*

The results of proximate analysis showed that *Nerita*

albicilla contains moisture, protein, fat, ash, fiber, carbohydrate of $12.45\% \pm 0.05\%$, $62.05\% \pm 0.10\%$, $5.58\% \pm 0.08\%$, $9.17\% \pm 0.03\%$, $6.60\% \pm 0.02\%$ and $4.15\% \pm 0.24\%$, respectively. It indicates that it is good source of protein. The results of amino acid analysis are presented in Table 1. The protein contained significant amount of branched-chain amino acids (BCAA) valine, leucine, isoleucine (a total of 187.8 mg g^{-1} protein) and lower content of aromatic amino acids phenylalanine, tyrosine and histidine (a total of 111.26 mg g^{-1} protein). This is suitable for treating liver cirrhosis [15, 16]. BCAA supplementation improves not only nutritional status, but also prognosis and quality of life in patients with liver cirrhosis. BCAA was also reported to prevent insulin-induced hepatic tumor cell proliferation [17].

The quality of protein is primarily a function of its essential amino acid composition. It is assessed by an amino acid or chemical score in which the amount of essential amino acid that is in the greatest deficit is expressed as a percentage of the amount present in a standard or reference protein. The results of amino acids score is presented in Table 2. The chemical score

Table 1 Amino acid composition of *Nerita albicilla*.

No.	Amino acids	mg g ⁻¹ protein*
Essential		
1	Histidine	30.41 ± 0.09
2	Arginine	30.89 ± 0.09
3	Threonine	44.91 ± 0.09
4	Valine	47.43 ± 0.09
5	Methionine	38.89 ± 0.25
6	Isoleucine	75.91 ± 0.16
7	Leucine	64.46 ± 1.61
8	Phenylalanine	36.64 ± 0.09
9	Lysine	53.83 ± 0.16
10	Tyrosine	44.21 ± 0.25
11	Cysteine	19.72 ± 0.09
Nonessential		
12	Aspartate	83.70 ± 0.09
13	Glutamate	143.22 ± 0.41
14	Serine	17.30 ± 0.09
15	Glycine	37.44 ± 0.25
16	Alanine	36.31 ± 0.25
17	Proline	26.97 ± 0.09

The average and standard deviation of three replicates

Table 2 Chemical score of *Nerita albicilla* amino acids.

Essential amino acids	Concentration in <i>Nerita albicilla</i> (mg g ⁻¹ protein)	FAO standard for adults (mg g ⁻¹ protein)	Chemical score
Isoleucine	75.91	40	100
Leucine	64.5	70	92.2
Lysine	53.8	55	97.8
Methionine + cysteine	58.6	35	100.0
Phenylalanine+tyrosine	80.7	60	100.0
Threonine	44.9	40	100.0
Valine	47.3	50	94.7

of *Nerita albicilla* protein was 92.2, represented by the score of leucine, which was the lowest among others. In addition, *Nerita albicilla* contained fiber of 9.17%. This composition exerts beneficial effect to patients with liver disease [16]. These findings support the traditional usage of *Nerita albicilla* to treat liver disease including cancer in Kei Island, Southern Maluku, Indonesia.

3.2 Extract of *Nerita albicilla* and Its Topoisomerase-I Inhibitor Activity

The yield of hexane, ethyl acetate and methanol crude extracts of *Nerita albicilla* were 2.05% ± 0.05%, 1.56% ± 0.06%, and 6.99% ± 0.14%, respectively. The crude extracts were made from 50.0 g DW and 100 mL solvent. The results of topoisomerase-I inhibitor activity using camptothecin as a standard are presented in Fig. 1. All extracts described topoisomerase-I inhibitor activity at concentration of 50 µg mL⁻¹. The inhibitor activity is grouped into catalytic and poison activities. The catalytic inhibitor activity is described by unchanged DNA supercoil after reacting with topoisomerase-I and its inhibitor (lane 6). The poison activity indicated by the formation of complex DNA/enzyme was shown in lanes 5, 7, 8. The hexane extract posed catalytic inhibitor activity while ethyl acetate and methanol extracts showed poison activity.

The methanol extract was chosen to determine the minimum inhibitory concentration (MIC) due to its highest yield. The results of minimum inhibitory concentration are presented in Fig. 2. The methanol extract showed inhibitory activity with a MIC of 2.5 µg mL⁻¹, camptothecin used as standard had a MIC of

Fig. 1 Inhibition of topoisomerase-I by various extracts of *Nerita albicilla* at concentration of 50 µg/mL. Lane 1: supercoiled DNA added with topoisomerase I. Lane 2: supercoil DNA added with DMSO 10%. Lane 3: supercoiled DNA, added simultaneously with solvent (DMSO 10%), and topoisomerase-I enzyme. Lane 4: relax DNA marker. Lane 5: supercoiled DNA added simultaneously with camptothecin at concentration of 34.84 µg/mL and topoisomerase-I. Lanes 6-8: the inhibition of topoisomerase-I in the presence of 50 µg/mL hexane, ethyl acetate and methanol extract, respectively.

100 µM (34.84 µg mL⁻¹). At concentration of 1.25 µg mL⁻¹ (lane 12), methanol extract did not inhibit topoisomerase-I activity, indicated by formation of relax DNA as shown by marker at lane 4.

The hexane (lane 5) and methanol extract (lane 7) of *Nerita albicilla* inhibit topoisomerase-I activity by interacting directly with the enzyme (catalytic). While ethyl acetate extract (lane 6) shows poison activity by formation of complex DNA/enzyme. The tested inhibitory concentration of hexane, ethyl acetate and methanol extract against topoisomerase-I was 50 µg mL⁻¹. In addition, the minimum inhibitory concentration of methanol extract was 2.5 µg mL⁻¹. Camptothecin (used as standard in this investigation) describes inhibitory effect at concentration of 34.84 µg mL⁻¹. This results indicated the potency of *Nerita albicilla* extract as topoisomerase-I inhibitor was much greater than camptothecin. Its activity as topoisomerase-I inhibitor might be due to an oxindole alkaloid isopteropodine contained by *Nerita albicilla* as supported by previous study [1, 4]. The presence of alkaloid compound was confirmed in this study (Table 3).

Camptothecin is an alkaloidal compound, first, it was isolated from the bark of a Chinese tree, *Camptotheca acuminata*. It was developed by National Cancer Institute (NCI), USA and has been commercially available as anticancer manufactured by

Fig. 2 Inhibition of topoisomerase-I by various concentration of methanol extracts. Lane 1: supercoiled DNA added with topoisomerase-I. Lane 2: supercoiled DNA marker. Lane 3: supercoiled DNA, added simultaneously with solvent (DMSO 10%), and topoisomerase-I enzyme. Lane 4: Relax DNA marker. Lane 5: supercoiled DNA added simultaneously with camptothecin at concentration of 34.84 µg/mL and topoisomerase-I. Lane 6-12. The inhibition of topoisomerase-I at concentration of 100.0 µg/ml, 50.0 µg/mL, 25.0 µg/mL, 12.5 µg/mL, 5.0 µg/mL, 2.5 µg/mL, 1.25 µg/mL, respectively.

Table 3 The chemical group of *Nerita albicilla* extract.

Tested compound	Solvent		
	Hexane	Ethyl acetate	Methanol
Carbohydrate	-	+	-
Protein	+	+	++
Amino acids	+	-	++
Alkaloid	-	-	+
Saponin	-	-	-
Flavonoid	-	-	-
Phenol hydroquinone	-	-	-
Triterpenoid	-	-	-
Steroid	+	++	+

Note : + (detected) ; - (non detected) ; ++ (higher intensity)

Aventis and Merck. It also has two derivatives approved by the FDA: Camptosar® (Irinotecan hydrochloride; CPT-11) for advanced colo-rectal carcinomas and Hycamtin® (Topotecan) for ovarian cancers. Topoisomerase inhibitor is also related to anticancer drug and anti-HIV agent such as betulinic acid which is a pentacyclic triterpenoid [18]. It was reported that it inhibited eukaryotic topoisomerase-I with IC₅₀ of 0.5 µM. Its derivative, dihydrobetulinic acid has been identified as anti-HIV agents for their inhibitory activity against HIV-1 replication in acutely infected H9 cells. They are also reported to be melanoma specific cytotoxicity agent against cell lines MEL-1, -2, -3 and -4. These compounds have been shown to completely inhibit tumor growth in athymic mice carrying human melanoma [18]. Marine alkaloid Lamellarin D (LAM-D) isolated from marine mollusk *Lamellaria* was also reported to have potent anticancer activity via inhibiting topoisomerase-1 [19]. The topoisomerase-I inhibitor content of *Nerita albicilla*

supported the rational of traditional usage to treat cancer disease.

3.3 Chemical Group of Topoisomerase I Inhibitor

The active extract of topoisomerase-I inhibitor was tested by its chemical group. The ethyl acetate extract contained carbohydrates (Table 3). Protein was detected in hexane, ethyl acetate and methanol extract, while free amino acids were contained by hexane and methanol extract. The extracts did not contain saponin, flavonoid, phenolic hydroquinone and triterpenoid. However, all extracts contained steroidal group. In addition, the methanol extract contained alkaloidal compound.

The rational of traditional usage to cure liver disease is suggested due to its chemical content of *Nerita albicilla*. Traditionally, *Nerita albicilla* is consumed by boiling in water and the water and flesh taken orally. Based on this practice, it is suggested that the compounds contributing to cure liver disease and cancer are steroidal alkaloid and branched chain amino acids. This was also supported by previous study [17, 19]. Furthermore, fulvoplumierin [6] that described virus inhibitor [8] might also contribute to cure hepatitis (viral infection). Isopteropodine and fulvoplumierin are suspected to avoid chronic hepatitis that cause cancer as both showed cytotoxic against various cancer and tumor cells [7].

4. Conclusions

The study concluded that *Nerita albicilla* contained

a good nutritional composition and other chemicals that support its traditional usage, especially to recover from liver disease. The investigation revealed that *Nerita albicilla* contains active compounds that could be potential for nutraceutical or pharmaceutical development. It is necessary to identify the active compounds and to conduct in vivo toxicological studies.

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