International Journal of Chemical and Biochemical Sciences
(ISSN 2226-9614)

Journal Home page: www.iscientific.org/Journal.html

Extraction of *Ulva lactuca* based on natural deep eutectic solvents (nades) and evaluation of its antioxidant activity

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Abstract

The green extraction process to get the active biologically substances derived from potential marine alga was a most effectively strategy. Since *Ulva lactuca* reported as promising source of bioactive substance, the optimization of extraction methode was should be done. An environmentally friendly extraction process has been carried out using various Natural Deep Eutectic Solvents (NaDES). This reasearch was aimed to find the total phenolic content in Ulva lactuca and evaluate the antioxidant activity. The air-drying and oven-drying methods were used to reduce the water content in *U. lactuca.* The extraction results showed that the air-drying treatment of *U. lactuca* extract based on NaDES 2 (Choline-chloride: Malic acid: H_2O (2:1:1)) produced total phenolics and inhibitory activity. Free radicals for DPPH and ABTS were higher than other NaDES extracts with a value of 48.20 ± 1.37 (mg GAE/mg, IC50 of 991.62 µg/mL for DPPH and 1609.07 µg/mL for ABTS. However, for the results over all NaDES-based extraction of *U. Lactuca* is still said to be less efficient than conventional organic solvents in producing total phenolics and antioxidant inhibitory activity.

Keywords: Antioxidants, Extraction, phenolics, NaDES, *U. Lactuca.*

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1. Introduction

Indonesia is a country with a high distribution and potential for marine biodiversity. One of the potentials is *U. lactuca*, which is a green macroalgae with a very fast growth rate and is easy to find along coastal waters [1]. In 2018, dried macroalgae was the leading commodity for Indonesian fisheries exports, with China accounting for 65% and the USA for 6% of the total value of dried macroalgae exports [2]. One candidate for use as a human nutritional supplement in the future is *U. lactuca*. The nutritional content of dried *U. lactuca* is quite high, such as carbohydrates (58.1%), water content (16.9%), ash (11.2%), protein (13.6%) and fat (0.19%) [3]. In general, *U. lactuca* is used as an alternative food ingredient and can be developed in various industrial fields. It is known that *U. lactuca* can be used as a source of medicine to treat various diseases such as cancer, malaria, inflammation, and diseases caused by viral infections [4, 5, 6]. Drying treatment at the beginning of harvest plays an important role in the quality of simplicial [7]. Various factors and drying methods such as the air drying process or using a drying device such as an oven can affect the quality of the sample and have their own advantages [8]. The drying process is carried out to reduce the water content, so that the simplicia does not rot easily. A good and correct drying process can maintain potentially active compound components so that they are not degraded [9]. Conventional organic solvents are widely used in the extraction process, but most of them have toxic properties [10]. One solution to obtain these active compounds is by using environmentally friendly solvents such as NaDES [11]. NaDES consists of a mixture of a hydrogen bond acceptor (HBA) and various hydrogen bond donors (HBD) at certain molar ratios [12]. (Pätzold *et al.*, 2019). Various types of NaDES have been used to extract important compounds from macroalgae [13]. *U. lactuca* has been widely known containing bioactive compounds, especially phenolic compounds. Most of phenolic compounds were antioxidant, apoptotic agents and participate in inhibiting Caco-2 cells [14]. However, there is

no information regarding the extraction of NaDES-based phenolic compounds from *U. lactuca* and their antioxidant activity. So this experiment discusses the NaDES-based *U. Lactuca* extraction process and evaluates its antioxidant activity.

2. Materials and methods

2.1 Material

U. lactuca was collected from Takalar waters, South Sulawesi Province (-5.456656, 119.392749) in 20 September 2022. Chemical used in this research were *choline chloride* (Sigma-Aldrich), D-(+)- *G lucose* (Merck), *Malic acid* (Merck), D - (-)- *F ructose* (Merck), Folin–Ciocalteu phenol reagent (Merck), gallic acid (99%) (Merck), distilled water, methanol pa. (Merck), dichloromethane pa. (Merck), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich), 2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), *t rolox* (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (Sigma-Aldrich), Na2CO3 (Sigma-Aldrich), and *96-well microplates*. The instruments used were including: a analytical scales, Philips HR - 211 blender, *centrifuge* (BIOBASE), *Rotavapor® R- 3 00, freeze-dryer Büchi Lyovapor* L-200, *Oven, Branson Ultrasonic* - 1800, Tecan-ELISA *reader,* Olympus CX23 Microscope, and other supporting equipment located at the BRIN CSC genomics laboratory, Cibinong-Bogor.

2.2 Methods

Post harvesting treatment of *U. lactuca* sample was the first step in this experiment. After preparation, the dryied of *U. Lactuca* was carried out using the air-air method and oven at 30 ºC. A water content test is carried out to ensure the water content after drying. *U.lactuca* extraction process using 3 types of NaDES and conventional solvents (methanol 80% and dichloromethane 100%). The analyzes tested included characterization of U. lactuca, water content, total phenolic content and antioxidant activity. Several type of NaDES with is components where used for *U. lactuca* can be seen in Table 1.

2.3 Preparation And Extraction U. lactuca

The raw materials *U. lactuca* newly obtained samples were washed with clean fresh water, then weighed 100 grams for each air drying and oven treatment at a temperature of 30 ºC. The drying process is carried out overnight for 3 x 24 hours, after drying it is weighed to calculate the water content. Dried simplicia was roughly chopped using a Philips HR - 211 blender to make the extraction process easier. The *U. lactuca* extraction process uses the *Ultrasonic Assisted Extraction* (*UAE*) method ± 20 minutes, 35 ºC using NaDES along with conventional organic solvents (methanol: water 80% (MeOH 80%) and dichloromethane 100% (DCM 100%)). The results of the extraction process are: The supernatant and filtrate obtained were separated using centrifugation for 5 minutes at 7000 rpm, then evaporated using *a Rotavapor ® R - 300.* Extract The samples obtained were frozen at -80 ºC for ± 24 hours then dried with a Büchi Lyovapor L-200 *freeze-dryer* to reduce the water content. The total phenolic content of NaDES was compared with extraction using commonly used conventional organic solvents [17, 18] and tested for antioxidant activity against DPPH and ABTS.

2.4 Testing Procedure

All tests include analysis of water content, total phenolic content, and antioxidant activity to determine the potential of *U. lactuca extract* NaDES based.

2.5 Water content analysis

Water content analysis to determine the percentage of water content contained in *U. lactuca* after drying using the air-air and oven method, whether it is in accordance with the quality standards that have been determined according to [19]. The analysis begins with drying the porcelain cup in an oven at 105 ºC for 1 hour, then weighing it. A total of 2 g of sample was put into a cup, dried in an oven at 120 ºC for 2 hours or until the weight was constant. Next, the cup was placed in a desiccator for ± 30 minutes and left to cool and then weighed again. The percentage of water content (wet basis) can be calculated using the formula:

$$\text{Water content (\%)} = \frac{B-C}{B-A} \times 100\%$$

Information:

A = Weight of empty cup (g)
B = Weight of cup filled with sample (g)
C = Weight of cup with dried sample (g)

2.6 Total Phenolic Content

Testing for total phenolic content refers to the method of Cahyaningrum *et al.* (2016) [20]. A standard curve was prepared with a gallic acid solution made in a series of dilution concentrations of 6.25, 12.5, 25, 50, 100, 200 µg/mL. Then, 5 mg of crude polyphenol extract was dissolved in 1 mL of ethanol. Each concentration of standard solution and crude polyphenol extract was taken 10 µl and then put into a 96-well microplate. Next, 50 µl of Folin-Ciocalteu reagent was added to the 96-well microplate and then incubated for 5 minutes. Next, 40 µl of 7.5% Na2CO3 was added and then incubated for 2 hours in the dark at room temperature. Absorbance readings were carried out at a wavelength of 750 nm. A standard curve was created by plotting concentration (µg/mL) versus absorbance (nm). The regression equation from the standard curve is y = ax + b, R2 = c, where x is the concentration and **y** is the absorbance. Total phenols are expressed in mg Gallic Acid Equivalents (GAE) per 1000 ppm extract.

2.7 Antioxidant Analysis

2.7.1 Test Antioxidant - DPPH (2.2-diphenyl-1-picrylhydrazyl) Method

Determination of antioxidant activity using the 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) method (Brand-Williams *et al*., 1995) [21] and modified by Zubia *et al.* (2009) [22]. The basic principle of the DPPH method is the reduction of DPPH radicals in methanol solution by the antioxidant H-donator (AH) to form the nonradical form of DPPH-H. In a 96-well microplate, 2 0 µl of each extract at various concentrations was mixed with 18 0 µl of DPPH solution (25 mg l^{-1}) so that final concentrations were 500, 250, 100, 50, and 25 (mg l^{-1}). The reaction was allowed to develop for 2 h in the dark at room temperature, and then the absorbance was read at 515 nm with a multi-well spectrophotometer (SunriseTM, TECAN). The DPPH concentration in the reaction medium was calculated from the calibration curve (n = 8; r = 0.99) determined by the following linear regression: [DPPH] = (Abs – 0.0398)/0.0137 to further deduce the

remaining percentage of DPPH (% DPPH). A curve of extract concentration against % DPPH was generated to estimate the extract concentration required to cause a 50% reduction from the initial DPPH concentration. This value is known as IC 50 (*Inhibition concentration*), namely the concentration of the sample solution needed to inhibit 50% of DPPH free radicals. This test was carried out in triplicate for each sample, and then the average value was used to calculate IC_{50} (µg/mL) . Ascorbic acid was used as a positive control and methanol as a negative control.

2.7.2 Antioxidant Test - ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) Method

Determination of antioxidant activity using the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) method [23], is the simplest and most commonly used method in various literature. ABTS• + (7 mmol L−1) and potassium persulfate (2.45 mmol L−1) were prepared separately and kept in the dark for at least 16 h and mixed. The ABTS radical solution was diluted with methanol to reach an absorbance value of 0.72 ± 0.02 at 734 nm. Each sample taken (25 µL) mixed with ABTS• + radical solution (125 µL) and stored in a dark place for 5 minutes. After incubation, the mixture was loaded into a 96-well plate and the absorbance was measured at 734 nm using a microplate reader. All measurements were performed in triplicate, and methanol was used as a negative control. Trolox was used as the reference standard, and a standard curve was constructed. The final result is expressed as IC_{50} (µg/mL) [24].

2.8. Data analysis

The experimental design used was a completely randomized design (CRD) with treatment factors differing in *U. lactuca* extraction methods. with various treatments using NaDES. The data obtained was processed using Microsoft Excel and the statistical product and service *solution* (SPSS) 26 application. The completely random design mathematical model refers to Steel and Torrie (1993) [25] with the following equation:

$$Yij = \mu + Ai + \varepsilon ij$$

Information

Yij =	*U. lactuca* extraction method treatment with various treatments using NaDES i-th level, j-th replication
µ =	Average value
Ai =	*U. lactuca* extraction method treatment with various treatments using NaDES level i
εij =	The influence of experimental error due to the influence of the treatment method of extracting *U. lactuca* with various treatments using the i-th level of NaDES and the j-th replication
i =	Test
j =	Treatment

Hypothesis of *U. lactuca extraction process* on total phenolic content and antioxidant activity are:

H0 :	*U. lactuca* extract based *N a D E S* had no effect on total phenolic content and antioxidant activity.
H1 :	Extraction of *U. lactuca* based *Na D ES has an* effect on total phenolic content and antioxidant activity.

3. Results and Discussions

3.1 Results

3.1.1 Morphology and anatomy U. lactuca

3.1.2 Water content U. lactuca

Characteristics and concentration of water content in *U. Lactuca* After drying using the air-drying and oven-drying method, it can be seen in Figure 2 and Table 1.

U. lactuca samples that have gone through preparation stages such as washing and drying using air and oven methods will be analyzed for their water content before being used in the next stage. Tests for water content from different drying results in this study can be seen in Table 1.

3.1.3 Extract characteristics U. Lactuca

3.1.3.1 Visual characteristics of the extract U. Lactuca

An extraction process for *U.lactuca* based on NaDES and conventional organic solvents has been carried out, which can be seen in Figure 3.

3.1.3.2 Yield of conventional solvent-based U. Lactuca extract

U. Lactuca Extract Yield with conventional organic solvents (MeOH 80% and DCM 100%) can be seen in Table 2.

3.1.3 Total Phenolic Content

The total content of phenolic compounds from *U. lactuca* was calculated based on the standard gallic acid line equation ($y = 0.001x – 0.002$; $R^2 = 0.998$). The total phenolic content was determined in all extracts obtained and the results are presented in Table 3.

3.1.4 Antioxidant Activity

3.1.4.1 Antioxidant Activity - DPPH (2.2-diphenyl-1-picrylhydrazyl) Method

The DPPH antioxidant test method for *U. lactuca* extract compared with the percentage of inhibition of the positive control ascorbic acid ($y = 4.839 x – 17.77$; $R^2 = 0.984$) can be seen in Table 4.

3.1.4.2 Antioxidant Activity - ABTS Method (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid))

The ABTS antioxidant test method for *U. lactuca* extract were compared with the percentage of control inhibition trolox ($y=9.557x-1.752$; $R^2 = 0.998$) can be seen in Table 6.

3.2. Discussion

Morphological form of *U. lactuca* (Figure 1A) In general, it is characterized by a light green to dark green color with flat talus and sheets with smooth but irregularly wavy edges. Kazi *et al.* (2016) [26] explained that the dominant color is green in *U. Lactuca* due to the presence of chloroplasts which almost fill the cell and have one to three pyrenoids.

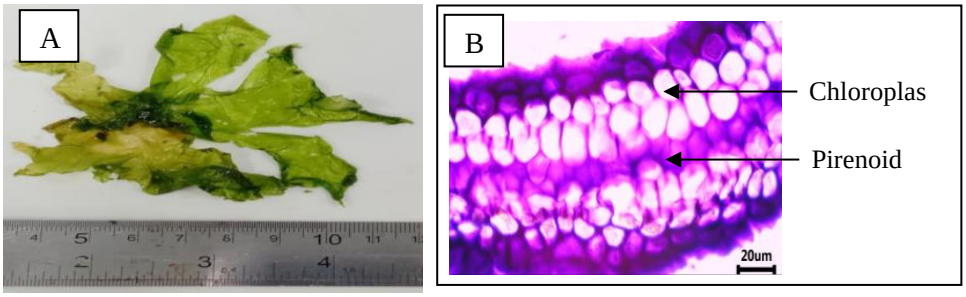

Figure 1: Morphology and anatomy *of U. lactuca*; (A) talus, (B) microscope observation with hematoxylin and eosin (H&E) staining of cross sections.

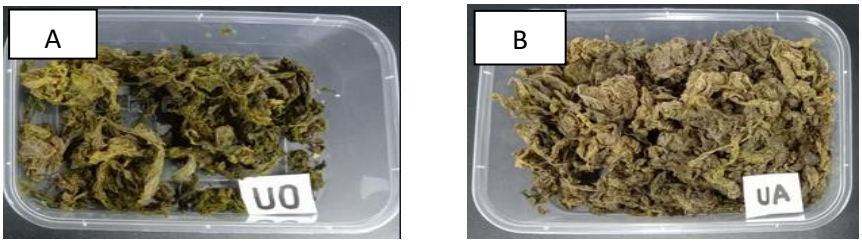

Figure 2: Samples of *U. Lactuca* (A) air-dried, and (B) oven-dried.

Table 1: Percentage of water content *of U. lactuca* in air and oven drying

Drying treatment	Gross weight (g)	Dry weight (g)	Average water content (%)
Windy	180	45.28	28.90 ± 7.11 [a]
Oven	125	26,24	19.63 ± 0.24 [b]

Note: the numbers followed by different *superscript letters* (*a*, *b*) *are significantly different (p<0.05)*.

(A)

(B)

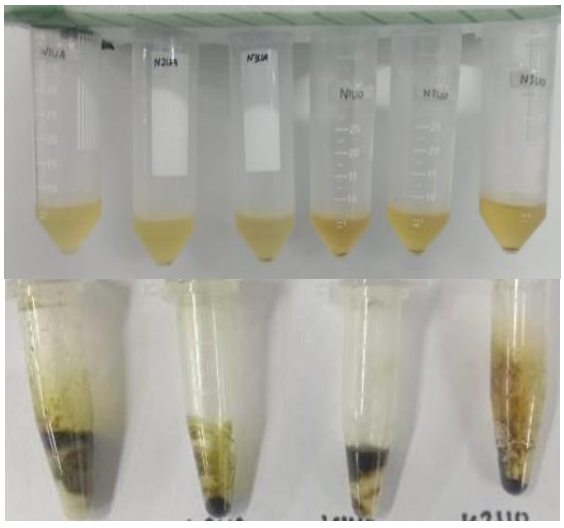

Figure 3: *U. lactuca* extract; (A) NaDES-based and (B) Conventional organic solvent-based.

Table 2: Yield of *U. Lactuca Extract* with conventional solvents (Me OH 80% and D CM 100%).

Treatment	Solvent	Code	Yield (%)
Air-drying	Methanol 80%	K1UO	12.67
	Dichloromethane 100%	K2UO	0.37
Oven-drying	Methanol 80%	K1UA	21.64
	Dichloromethane 100%	K2UA	0.24

Table 3: Results of total phenolic content of *U. lactuca* extract

Treatment	Solvent	Code	mg GAE/mg sample
Air-drying	CH Cl : Glu : H_2O	N1UA	47.50±0.66 [a]
	CH Cl : Ma : H_2O	N2UA	48.20±1.37 [a]
	CH Cl : Fru : H_2O	N3UA	45.97±0.85 [a]
	MeOH 80%	K1UA	51.43±1.52 [a]
	DCM 100%	K2UA	63.83±6.30 [b]
Oven-drying	CH Cl : Glu : H_2O	N1UO	45.13±1.18 [a]
	CH Cl : Ma : H_2O	N2UO	45.60±0.36 [a]
	CH Cl : Fru : H_2O	N3UO	45.40±0.61 [a]
	MeOH 80%	K1UO	47.97±1.19 [a]
	DCM 100%	K2UO	92.30±0.53 [b]

Note: Numbers with different superscript letters (a,b) are significantly different ($p < 0.05$).

Table 4: Antioxidant activity of the DPPH method of *U. lactuca* extract

Treatment	**Solvents**	**Code**	**IC_{50} (µg/mL) DPPH**
Air-Drying	CH Cl : Glu : H_2O	N1UA	5404
	CH Cl : Ma : H_2O	N2UA	991.62
	CH Cl : Fru : H_2O	N3UA	1371.29
	MeOH 80%	K1UA	2537.15
	DCM 100%	K2UA	857.5
Oven-Drying	CH Cl : Glu : H_2O	N1UO	2602.5
	CH Cl : Ma : H_2O	N2UO	3570
	CH Cl : Fru : H_2O	N3UO	38300
	MeOH 80%	K1UO	771.9
	DCM 100%	K2UO	263.82

Table 5: Antioxidant activity of the ABTS method of *U. lactuca* extract

Treatment	**Solvents**	**Code**	**IC_{50} (µg/mL) ABTS**
Air-Drying	CH Cl : Glu : H_2O	N1UA	1639.17
	CH Cl : Ma : H_2O	N2UA	1609.07
	CH Cl : Fru : H_2O	N3UA	1637.07
	MeOH 80%	K1UA	554.14
	DCM 100%	K2UA	404.19
Oven-Drying	CH Cl : Glu : H_2O	N1UO	1649.93
	CH Cl : Ma : H_2O	N2UO	1616.4
	CH Cl : Fru : H_2O	N3UO	1647.03
	MeOH 80%	K1UO	1544.9
	DCM 100%	K2UO	260.15

U. Lactuca width found ranged ±8 cm and attached to the rock substrate. This is due to the habitat of *U. Lactuca* 44% is located in sea bays and 34.4% in coastal rock formations, so it is most commonly found in these areas [27]. H&E Imaging *of U. Lactuca* in cross section (Fig. 1B) shows the presence of eosin which colors the cell membrane pink which is known as chloroplast and the presence of purple hematoxylin which is known as pyrenoid. Bar-Shai *et al.* (2021) [28] in their research used H&E to color cell nuclei. The thallus cells in *U. lactuca* are oval in shape and the characteristic chloroplasts and pyrenoids are visible. In general, there are more than one chloroplasts in the outer layer of the pyrenoid that are stacked on top of each other. The visual appearance of dried *U. Lactuca simplicia* (Fig. 2) shows that water content plays a very important role in maintaining the quality obtained. Simplicia *U. Lactuca* with air-drying treatment (Figure 2A) has a paler color compared to simplicia *U. Lactuca* with oven-drying treatment (Figure 2B). This is due to the hygroscopic nature of macroalgae, so the drying process greatly affects the physical and chemical condition of macroalgae [29]. The type of solvent and extraction method used greatly influence the results of the *U. Lactuca extraction process*, both physically and chemically (Fig. 3). The characteristics of the NaDES-based *U. lactuca* extract from (Fig. 3A) have a greenish yellow visual appearance and a slightly liquid gel-like texture. This is because the mixture of HBA and HBD that forms NaDES affects the viscosity properties, so that the extract is not in powder or solid form [30]. Conventional solvent-based *U. Lactuca* extract (MeOH 80% and D CM 100%) (Fig. 3B) has a texture resembling a coarse paste. Extraction efficiency to obtain target bioactive compounds is greatly influenced by various conditions, the type of solvent being very important as a parameter for isolating a target bioactive compound [31].

Extract yield results with conventional organic solvents (MeOH 80% and DCM 100%) (Table 2) show that the yield of K1UA extract has a higher percentage with a value of 21.64%, meanwhile from extract K2UA has the lowest yield with a value of 0.24%. This is because the K1UA extract solvent is extracted using 80% MeOH which has more polar properties compared to the K2UA extract which is extracted using DCM 100%. The high polarization properties of methanol can dissolve polar and semi-polar compounds so that it has a high yield [31]. Extraction of phenolic compound components was carried out on the marine macroalgae *U. lactuca* using different NaDES and conventional solvents (MeOH 80% and DCM 100%) as controls (Table 3). The N2 UA extract had the highest value for total phenolic content showing a value of 48.20 ± 1.37 mg GAE/mg sample ($p > 0.05$). The K2UO extract had the highest total phenolic content with a value of 92.30 ± 0.53 for the whole *U. Lactuca extract.* The overall extract obtained from NaDES has a low value compared to the use of conventional solvents (MeOH 80% and DCM 100%). This is in accordance with the statement from Obluchinskaya *et al.* (2019) [13] who explained that the NaDES-based extraction results were characterized by a relatively low total content of polyphenolic compounds such as phlorotannin compared to extraction using the conventional organic solvent 70% acetone. Based on a completely randomized factorial design analysis with two factors, namely differences in drying treatment and the type of solvent used, it is known that the use of NaDES and the control solvent methanol 80% are not significantly different. In line with the total phenolic content, N2UA extract has the best DPPH free radical scavenger activity with a value of IC_{50} 991.62 µg/mL (Table 4). K2UO extract has the highest DPPH inhibitory activity among all *U. lactuca* extracts with value 263 .82 µg/mL. DPPH free radical inhibitory activity value of the whole *U. lactuca* extract still relatively weak.The free radical scavenger activity of ABTS is also in line with the total phenolic content obtained as well as the IC_{50} value. DPPH free radical scavenging activity (Table 5). N2UA extract has stronger activity compared to other *U. lactuca* extracts which are extracted based on NaDES with a value of 1609.07 µg/mL. Meanwhile, for all *U. lactuca* extracts, K2UO extract had the highest ABTS free radical inhibitory activity with a value of 260.15 µg/mL. Sedjati *et al.* (2019) [32] explained that the lower the $IC_{50,}$ the higher the antioxidant activity. Molyneux (2004) [33] explains that the antioxidant activity of a compound can be divided into several categories, namely very strong if the IC_{50} value extract is <50 µg/mL, strong 50-100 µg/mL, medium 101-150 µg/mL and weak >150 µg/mL.

4. Conclusions

U. lactuca extract NaDES-based has a relatively low total phenolic content and antioxidant activity compared to conventional organic solvent-based *U. lactuca* extracts.

5. Acknowledgement

The author would like to thank the BRIN Talent Management, BRIN Vaccine and Drug Research Center and Bilateral Exchange ***DGHE*** - ***JSPS*** Joint Research Projects, IPB.

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