Natural reagent from Secang (*Caesalpinia sappan* L.) heartwood for urea biosensor

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Natural reagent from Secang (*Caesalpinia sappan* L.) heartwood for urea biosensor

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Abstract. A simple and environmental friendly method have been developed for urea biosensor development. Secang (*Caesalpinia sappan* L.) heartwood has been extracted using pure water and ethanol-HCl. The extracts were then characterized their properties and stability for further application. The urea biosensor detection was based on the reaction of urea with urease resulted in ammonium ion which could change the colour of *Caesalpinia sappan* L extract. The colour changes with the urea concentration were determined using spectrophotometer UV-Vis. The results the water and ethanol-HCl extract of *Caesalpinia sappan* L extract were stable at pH 6 and the addition of reducing agent. The ethanol-HCl was more stable on the heat treatment (40°C, and 60°C) compare to water extract. Furthermore, the correlation between colour change and urea concentration using extracts showed the ethanol-HCl extract has higher sensitivity (y = 106,01x - 0,264 with value R^2 = 0,962) than that of water extract (y = 96,957x + 0,482 with value R^2 = 0,955).
Keywords: biosensor, natural reagent, secang, urea analysis.

1. Introduction

Urea analysis is one of the important parameters in the health general checkup, which the urea level in the blood reflect the kidney and liver disease. Indonesia is one of the high rate countries with kidney failure. World Health Organization estimates that kidney failure patients in Indonesia will be increased in 2025 by 41.4%. The patients with kidney failure were 70,000 in 2011 and will continue to increase by around 10% annually [1]. Basic Health Research (Riskesdas) in 2013 also noted that there were 5 diseases with the highest prevalence in Indonesia which spread in provinces in Indonesia, one of them was liver disease.

The important of kidney failure early detection stimulate researcher to develop fast and accurate analysis methods for determining the urea levels in the blood. One of the remarkable analytical device to detect urea is biosensors. Biosensor combines a biological sensing element (specific reacted with analyte) with a transducer (convert the biological response to readable signal). The biosensors were widely studied to detect important molecule related to human disease such as glucose [2], uric acid [3], albumin [4], urea [5] and cancer marker [6]. The biosensor development could be performed in the biological sensing or on the detection system. The biosensor detection system widely studied is colorimetry. Colorimetry is a method of chemical analysis based on a comparison of the intensity of colours formed with the colour of a standard solution. In simple terms it can be assumed that colorimetry

is a measurement of concentration based on the colour difference comparison. This colorimetry method involves the colour production from the redox indicator, chemical reaction, acid-base indicator or natural based indicator.

Indonesia rich of plants recognized as natural dyes as food, beverage, cloth and furniture colouring agent. One of the Indonesians well known natural dyes is the secang (*Caesalpinia sappan* L.) heartwood. The chemical compound found in the *Caesalpinia sappan* L. is brazilein. Brazilein family consist of brazilin, brazilein and 3'-O-methylbrazilin subtypes (**Fig. 1**) [7].

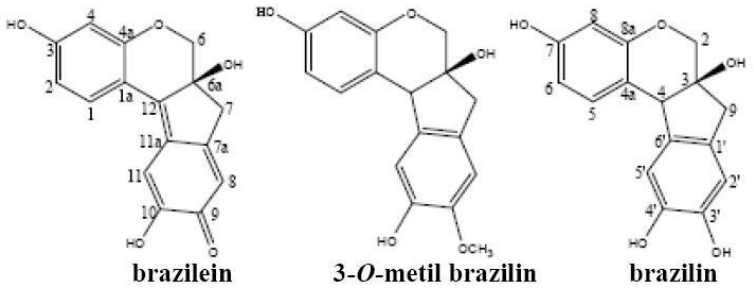

Figure 1. Brazilein family structure found in the *Caesalpinia sappan* L [7].

This research used natural dyes *Caesalpinia sappan* L. as an indicator in urea biosensors. Characterization of the dyes studied including pH stability, reducing-oxidizing agent effect, temperature effect and storage stability. The use of the dye in urea biosensor was based on the urea reaction using the urease to produce ammonia would change the colour of the dye linearly with the concentration of the urea.

2. Materials and Methods

2.1. Materials

Secang (*Caesalpinia sappan* L.) heartwood was collected from local market (Purwokerto, Central Java, Indonesia). Ethanol absolute, disodium hydrogen phosphate, sodium citrate, sodium dihydrogen phosphate, sodium acetate, hydrochloric acid 36%, citric acid and acetic acid were purchased from Merck Millipore (US). L-Ascorbic acid (reagent grade) and Urease (from *Canavalia ensiformis*, type III) were from Sigma Aldrich (US). All or reagent were analytical grade except mention separately.

2.2. Apparatus and Measurements

UV-Vis spectrophotometer (Shimadzu UV-1800, Japan), blender (Miyako BL-152 GF), hotplate stirrer (Thermoline Cimarec 3, US), micropipette (Dragonlab, China), tip (Biologix), pH meter (Hanna instruments), analytical balance (Acis) and laboratorium glassware (IWAKI Pyrex).

2.3. Secang (Caesalpinia sappan L.) heartwood extraction

Secang heartwoods were cut into small pieces using scissor and blender. The secang heartwood was then extracted using two different solvents in separate extraction flash of pure water and ethanol-HCl (ethanol 96% and HCl of 1.5 N ratio of 85: 15). The heartwood samples 20 g was added to 100 mL of solvents and mix with magnetic stirrer for 1 hour. The mixture was then precipitated for 30 minutes and filtered with filter paper. The filtrate was measured the colour intensity using a UV-Vis spectrophotometer at 400-700 nm. The filtrate was diluted using the solvent when the absorbance was greater than 0.8.

2.4. pH effect on the colour intensity of extract

Secang extract of 2 mL dissolved in 100 mL buffer (citric buffer for pH 3, acetic buffer for pH 4-5, phosphate buffer for pH 6-7 and tris-HCl buffer for pH8). The extract – buffer ratio depends on the absorbance obtained of 0.8. When the absorbance is too low, the buffer ratio was decreased and vice versa. The diluted extract was then measured at 400 - 700 nm. The absorbance of different pH condition of the extract was then compared.

2.5. Oxidizing and reducing agent effect study on the colour intensity of extract

Diluted extract of 1 mL in test tube was added 0.1 mL of 0.1 M oxidizing agent of hydrogen peroxide. Another 1 mL of extract was added 5 mg reducing agent of ascorbic acid. The mixture was then kept in room temperature (25°C) for two hours. Oxidizing and reducing effect observed by measuring the colour every five minutes. The colour decreasing of the extract was then recorded.

2.6. Heat treatment effect study

Secang extract of ten mL was kept in closed test tube. The stability of the extract colour with temperature change were studied at room temperature (about 25°C), at 40°C and at 60°C. The colour changes were measured at 1 h and 2 hrs storage duration.

2.7. Secang (Caesalpinia sappan L.) extract study as urea biosensor indicator

Urea standard solution was prepared in pure water with the concentration range 1 to 5 mM for water-based extract and 2 to 10 mM for ethanol-HCl based extract. These concentration ranges were preliminary studied before to achieve the absorbance of 0.2 to 0.8. Five mL of standard urea solution was mix with 10 unit of urease in phosphate buffer. The enzymatic reaction was performed at room temperature for 15 minutes. Indicator of 1 mL secang extract was added to the urea mixture and the colour was measured at 400 – 700 nm. Calibration curve of urea concentration and absorbance were then preparing to get the linear responses.

3. Results and Discussion

3.1. Caesalpinia sappan L. heartwood extraction

This study was started with the identification of materials, "secang" heartwood at the Biology Laboratorium, Faculty of Biology, Jenderal Soedirman University, Purwokerto. The identification results confirmed that the "secang" used was a species of *Caesalpinia sappan* L.

The secang water-based extract was red-orange and ethanol-HCl extract was bright red solution (Fig. 2). The water extract needs 10 times dilution whereas the ethanol-HCl extract needs 20 times dilution to achieve the appropriate absorbance (about 0.8). The ethanol-HCl extract showed two maximum wavelengths of 445 and wavelength of 518 nm, thus the visual bright red solution may due to the combination of red and yellow. In another side, the water based diluted secang extract showed a similar peak as ethanol-HCl based extract of two peaks at 446 nm and at 538 nm. The higher wavelength of the second spectrum resulted in more yellow-orange of the extract.

Ethanol-HCl solvent gives better results for extraction of secang stems compared to water, shown in the higher dilution ratio of ethanol-HCl extract, which means, the ethanol-HCl was more concentrated. The better ethanol-HCl solvent for extraction may due to the lower polarity of ethanol-HCl than the water polarity. Furthermore, the HCl in ethanol made acidic condition promoted the cell wall disturbed and easily extracted. Furthermore, the acidic condition of HCl was more effective than acetic acid in plant pigment extraction [9]. The main *Caesalpinia sappan* L. extract red pigment constituent is brazilin, which also known as biological staining [10]. The oxidized form of brazilin, namely brazilein was also found in the extract, mainly when organic solvent used and extraction process expose to oxygen [10].

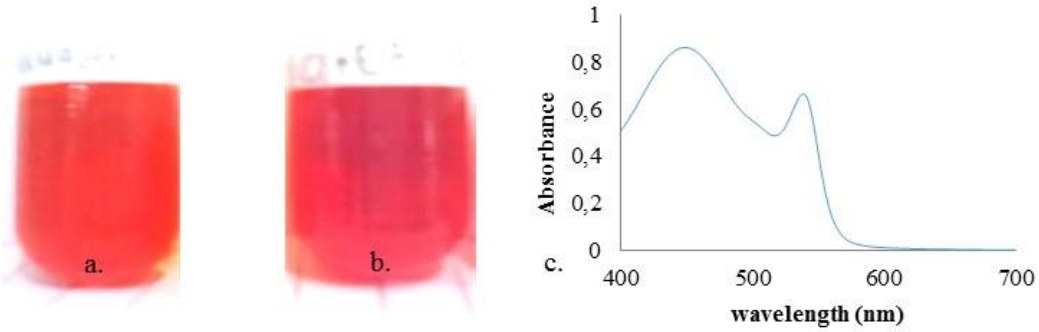

Figure 2. *Caesalpinia sappan* L. water extract (a) and ethanol-HCl extract (b), with two peak measured by spectrophotometer (c).

3.2. Stability study

The extract colour stability study was to determine the characteristic of extracts against pH, oxidizing-reducing agents, heat treatment, and storage. These parameters would be important for future application of the extract as natural dye.

3.2.1. pH effect. Brazilin was the major compound in the secang heartwood extract as biosensor indicator studied. The brazilin could be transformed into several ion form, depend on the pH of the solution. In this work, the extract colour was studied in the range pH of 3.0 to 8.0, the wide range of pH including acidic, neutral and alkaline condition. The result (**Fig. 3**) showed that the increasing absorbance of the heartwood extract with the increasing of pH. The increasing absorbance significantly observed at 539 nm. The visual colour change from light yello to pink for both of *Caesalpinia sappan* L. water extract and ethanol-HCl extract.

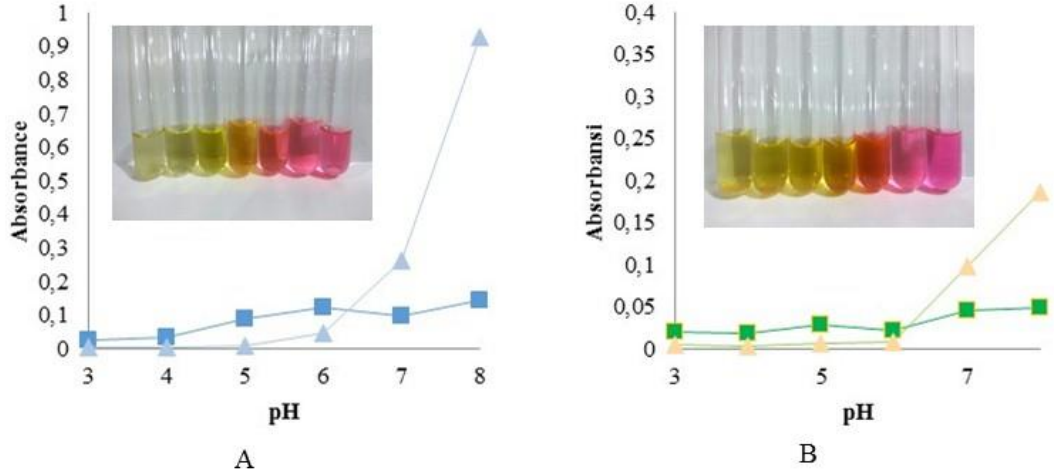

Figure 3. Effect of pH on *Caesalpinia sappan* L. water extract (A) and ethanol-HCl extract (B) at 445 nm (square marker) and 539 nm (triangle marker). Inset the visual of *Caesalpinia sappan* L. extract at different pH of 3.0 to 8.0.

The *Caesalpinia sappan* L. extract colour change with pH has been previously studied and reported [8], as diprotic acid ionized in water solution as follows:

$H_2Y \leftrightarrow HY^- + H^+$

$HY^- \leftrightarrow Y^{2-} + H^+$

The presence of H_2Y, HY^- and Y^{2-} in the solution related to the structure change of brazilin and brazilein (**Fig. 4**). The increasing of pH promoted H+ releasing from hydroxyl group of benzene ring of brazilin and brazilein. Brazilin dissociation perform in three steps, whereas brazilein dissociation only two steps process.

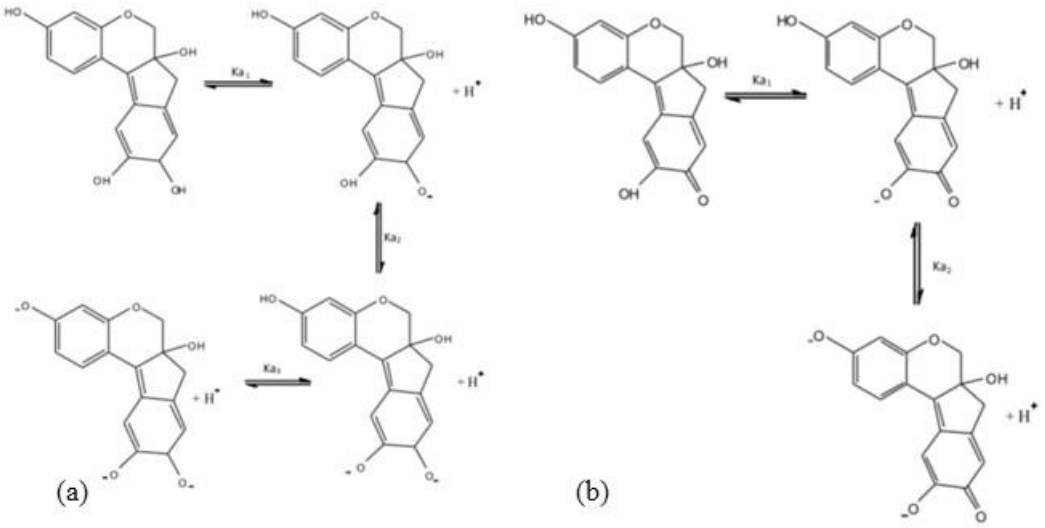

Figure 4. Brazilin (a) and brazilein (b) ionization [7]

3.2.2. Oxidizing and reducing agent effect. The colour change of the *Caesalpinia sappan* L. extract was observed by the addition of ascorbic acid as an example of reducing agent and hydrogen peroxide as oxidizing agent. In general, the oxidizing and reducing agent reduce the absorbance of the extract **(Fig. 5)**, however oxidizing agent increase the absorbance when measured at 550 nm. The reducing of extract absorbance may due to the oxidizing agent attack the red colour flavylium to lose their proton, resulted colourless solution [8], and the redox reaction of some anthocyanin are irreversible [9].

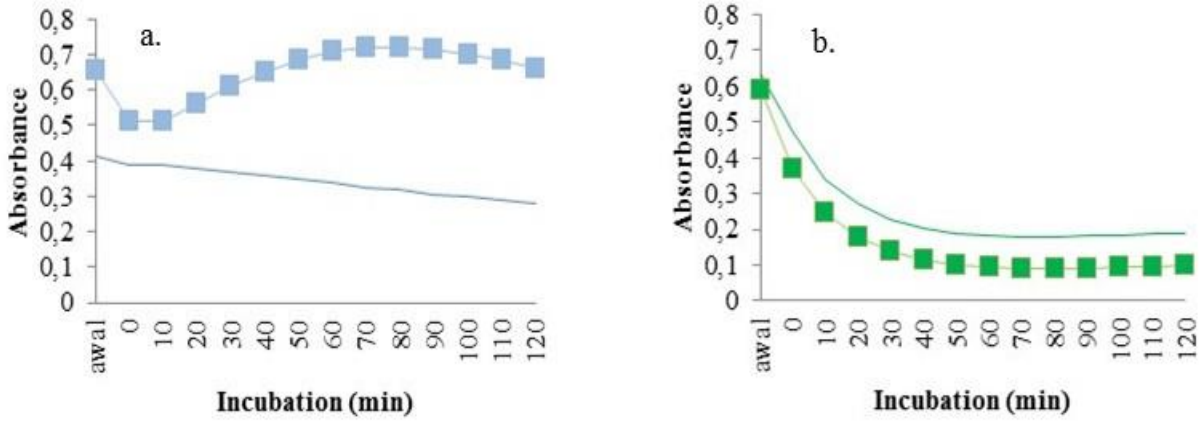

Figure 5. Effect of oxidizing (hydrogen peroxide) (a) and reducing (ascorbic acid) (b) agent on the *Caesalpinia sappan* L. extract absorbance with the incubation duration, measured at 450nm (triangle marker) and 550 nm (square marker).

3.2.3. Heat treatment effect. The natural extract colour stability could be changed with temperature due to the anthocyanin stability. This study observed the *Caesalpinia sappan* L heartwood extract treated at 40°C and 60°C for one and two hours, in the closed dark containers. The higher temperature and longer

incubation time decreased the extract absorbance of ethanol extract (**Fig. 6**). In another side, the *Caesalpinia sappan* L water extract showed increasing absorbance with the high temperature and longer treatment. The absorbance decreasing showed the brazilin degradation which was related to the increasing of the temperature [10, 11].

3.2.4. Urea biosensor study. Urea biosensor study was performed using urease enzyme to catalyse the urea breakdown into ammonium and carbon dioxide (1). The resulted ammonium ion (2) would change the extract colour related to the urea concentration such described in the extract colour change with the effect of various pH. The result of series urea standard solution of 1 to 5 mM showed linear responses measured using spectrophotometer at 539 nm (**Fig. 7a**) for water extract of *Caesalpinia sappan* L heartwood. In another side, the ethanol extract showed linear responses of 2 to 10 mM (**Fig. 7b**). The *Caesalpinia sappan* L water extract showed lower linear range and sensitivity, whereas the ethanol extract showed wider linear range with higher sensitivity.

$$(NH_2)_2CO + 3H_2O \xrightarrow{urease} CO_2 + 2\,NH_4OH \quad (1)$$

$$NH_4OH_{(aq)} \leftrightarrow NH_4^+{}_{(aq)} + OH^-_{(aq)} \quad (2)$$

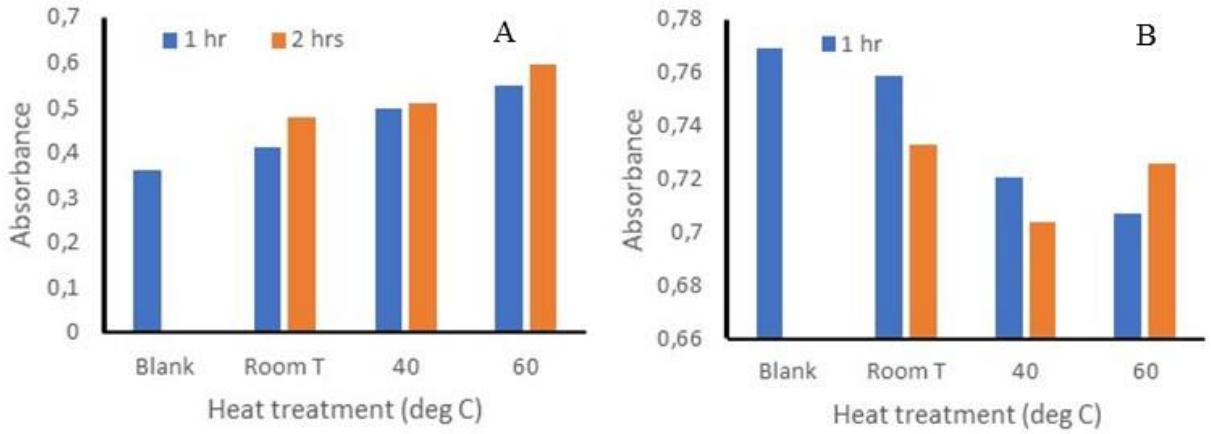

Figure 6. Effect of heat treatment on the *Caesalpinia sappan* L. water extract (A) and ethanol-HCl extract (B) absorbance measured at 450nm for one and two hours.

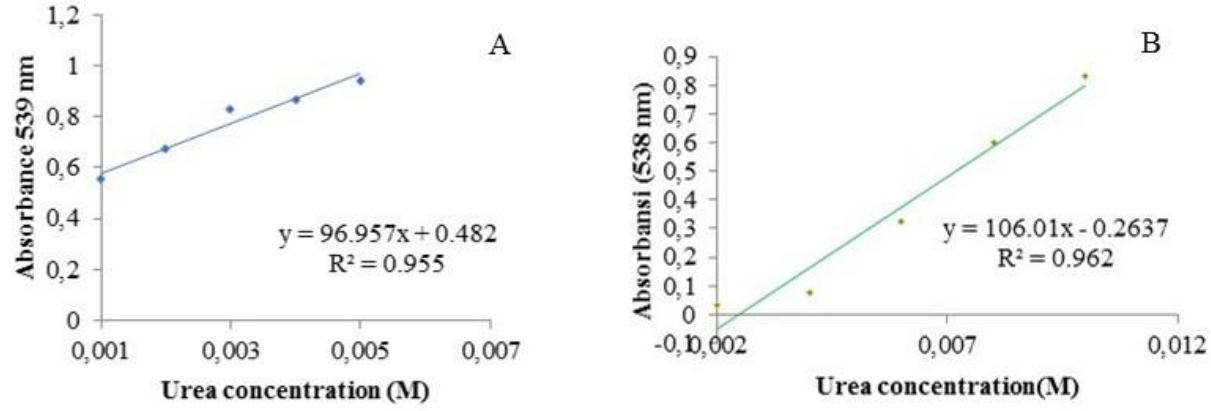

Figure 7. Calibration curve of urea biosensor using *Caesalpinia sappan* L water extract (A) and ethanol-HCl extract (B) as indicator.

4. Conclusion

Caesalpinia sappan L heartwood water and ethanol-HCl extract showed great performance as urea biosensor indicator as one of a green chemistry approach in analytical method development. The urea detection study showed linear responses of 1 to 5 mM using water extract and 2 to 10 mM using ethanol-HCl extract. Furthermore, the *Caesalpinia sappan* L extract characterization was performed for further application.

Acknowledgement

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