Evaluation of Roselle (Hibiscus sabdariffa L.) Calyx Extract Stability for Intestinal Nematode Egg Staining

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Abstract. Roselle (Hibiscus sabdariffa L.) calyx extract is rich in anthocyanin and it has been widely used as a natural colouring agent, for example as an alternative dye for eosin 2% in intestinal nematode egg staining. However, as a natural colour, the temperature and storage time are often altered anthocyanin activity. Therefore, the objective of this study was to investigate stability of Roselle calyx extract using various time and temperature storage and its staining properties on the intestinal nematode eggs. The Roselle calyx was extracted using maceration technique for ± 3 days and then made 80% concentration of extract. Extracted is stored within 1, 7, 14, and 21 evaluation days in room temperature (25°C) and cold temperature (4°C) for extract characterization, absorption of anthocyanin, pH, and staining observation. The result showed that natural extract from Roselle calyx stained intestinal nematode eggs and stable within 21 days storage in cold temperature (4°C).

1. Introduction

Tropical climate conditions and high humidity cause high cases incidence of intestinal worms in Indonesia. Worms were transmitted through worm eggs which are released together by the faeces of an infected person. In areas that don't have adequate sanitation, these eggs will pollute the soil. Four most common worm species infecting humans are roundworms (Ascaris lumbricoides), whipworms (Trichuris trichiura), and anthropophilic hookworms (Necator americanus and Ancylostoma duodenale) [1]. The process of identifying worm eggs is one of important steps for the diagnosis of intestinal worms and the treatment that will be carried out. Worm eggs must be identified and taken from the faeces of an infected person [2].

Microscopic examination of worm eggs from faeces consists of qualitative and quantitative examination. Qualitative examination can be done by direct slide method, floating method, modification of meryolate iodine formaldehyde method, cello tape method, concentration method, thick smear technique cellophane covered and formal ether sedimentation methods. For quantitative examination, can be used stoll method and kato katz method [3]. Qualitative examination of worm eggs by direct slide method with eosin dye 2% was most often used because the process was easy and very fast. However, in the examination process, especially for mild infections, it is difficult to identify worm eggs so that proper staining is needed to identify the worm eggs.
Eosin 2% dyes are most commonly used by medical clinics and hospital laboratories as a microscopic examination of faeces for identification of worm eggs and other impurities, but Eosin 2% has the disadvantage, such as expensive, hard to decompose, toxic for environment because of waste product, and flammable. Therefore, we need alternative solution of a 2% eosin for worm egg staining that natural dyes, organic and eco-friendly, such as the use of anthocyanin derived from Roselle calyx extract [4].

Roselle (Hibiscus sabdariffa L.) calyx is a plant that grows in tropical and subtropical regions, and is widely cultivated in Indonesia. Roselle has a single flower in the armpit of the leaf and is red, the red colour of the Roselle comes from anthocyanin. Anthocyanin in rosella flowers have been used for dyeing textiles, food, pharmaceuticals, the cosmetics industry, as indicators of acid base, and histology staining, anthocyanin dyes from rosella flowers are obtained by extraction techniques [4].

Roselle flower extract has been widely used as a natural colouring, one of which is the use as a natural alternative coloration for the preparation of large red chilli plants. Furthermore, Roselle flower are also chosen based on their availability in nature and the ease of obtaining. This flower also has been known by the public to have many health benefits [4].

Fresh extract of 80% rosella calyx can be used for examination of worm eggs as a substitute for eosin 2% [5]. Roselle petals have also been shown have anthocyanin natural dyes, and will be stable if stored within 1 day [6]. Until now, it is not yet known how the effect of storage time more than 1 day for the roselle extract and how storage temperature can affect the stability of rosella flower extract. Furthermore, researcher will evaluation all process by identification of worm egg staining with various storage day and temperature. Therefore, the objective of this study was to investigate stability of Roselle calyx extract using various time and temperature storage and its staining properties on the intestinal nematode worm eggs.

2. Material and Method

2.1. Sample preparation of 80% Roselle (H. Sabdariffa) calyx extract
Dried Roselle calyces were obtained from the local flower store in Bandung, Indonesia. The calyx has been conducted for determination at School of Technology and Life Sciences, Institut Teknologi Bandung, Indonesia. Roselle calyces were cut into small pieces and put in a container for maceration process by adding 3000 ml of distilled water. After 3 days of incubation, the extract was filtered and concentrated using evaporation method by Buchi rotatory evaporator. The extract is then made into a solution with a concentration of 80% by adding distilled water.

2.2. Stability test
The solution of 80% roselle calyx extract was stored in a dark bottle at various temperature (room temperature, 25°C and cold temperature, 4°C). Solution in each of temperature divide into 3 bottle various days (1, 7, 14, and 21 days) experiment.

2.2.1. Evaluation of extract colour and intensity: Dropped 80% roselle calyx extract solution into petri dish, then observed color and intensity directly

2.2.2. Absorbance of anthocyanin concentration and pH measurement. The solution of 80% Roselle calyx extract was measured for wavelength in the range of 400 – 800 nm by UV / Visspectrophotometer(JENWAY 6705) then find the wavelength for anthocyanin concentration. For measuring the absorbance in various temperature and storage days, dilute 80% Roselle calyx extract into 10% concentration then measured the absorbance using 600 nm wavelength. For pH measurement was used Mettler Five Easy F20 pH meter.

2.2.3. Microscopic observation using worm egg. Solution of 80% Roselle calyx extract was dropped on the intestinal nematode eggssuspension (Ascaris lumbricoides and Trichuris trichiura), then
covered the slide with cover glass. The slide was examined under light microscope (Olympus CX23) with 40x magnification. Grading and scoring of extract staining on intestinal nematode egg worms was verified by 2 parasitology experts. Score 1 means undefined and unstained, score 2 means well defined and stained.

3. Result and Discussion
In this study, 80% rosella (*Hibiscus sabdariffa* L.) calyx extract solution was tested for stability with various days (days 1, 7, 14, and 21) and various storage temperatures (room temperature, 25°C and cold temperature, 4°C). Furthermore, an evaluation of the stability of the extract was carried out, including observations of colour, intensity, absorbance of anthocyanin concentration, pH measurement and microscopic observation of nematode eggs.

| Days | Temperature | Colour | Intensity |
|------|-------------|--------|-----------|
| 1    | 25°C        | Red    | +++       |
|      | 4°C         | Red    | +++       |
| 7    | 25°C        | Red    | +++       |
|      | 4°C         | Red    | +++       |
| 14   | 25°C        | Red    | +++       |
|      | 4°C         | Red    | +++       |
| 21   | 25°C        | Red    | +++       |
|      | 4°C         | Red    | +++       |

+: Light colour, ++: Moderate colour, +++: Deep colour

The extract was made by maceration method using a water solvent for anthocyanin binding [8]. Furthermore, evaporator method was used to concentrate the extract, then dissolved to reach an 80% concentration which is the optimum concentration that can be used to observe nematode eggs as a substitute for eosin 2% [5].

Figure 1. Absorbance of 80% Roselle calyx extract at various temperature and days storage.
Based on observations of colour intensity from days 1 to 21, the extract have consistent red colour both at room temperature and cold temperature (Table 1). Red colour indicates presence of anthocyanin pigment, which is the main pigment in Roselle calyx extract [7]. Anthocyanin contains delphinidin-3-siloglucoside, delphinidin-3-glucoside, cyanidin-3-siloglucoside, while the flavonoid contains gosipetin and mucilago (rhamnogalakturonan, arabinogalactan, arabinan) [8].

The presence of consistent red colour days 1 to 21 support absorbance result to show anthocyanin concentration for 21 days at room temperature and cold temperature (figure 1). Based on the ANOVA test, it showed a significant difference between the absorbance values of extracts stored in cold temperatures higher than room temperature (Figure 2). The high absorbance value of extract solution at cold temperatures indicated high levels of anthocyanin [9]. Anthocyanin storage at 6°C is the best condition compared to temperatures of 18.3°C and 37.2°C. Storage in cold temperature have important effect, because of: 1) co-pigmentation reactions, 2) Polyphenolase enzyme content that is still present in the extract, that can catalyse browning reactions, which will be inhibited by storage in cold conditions [6].

pH measurement from days 1 to 21 shows that the pH value at cold temperatures is higher than room temperature (figure 2). pH value of the 80% Roselle calyx extract solution that stored at room temperature or cold temperature have acidic pH range of 2-3, because of the evaporation process causes water decreasing in extract that can be increase acid concentration for triggering pH decreasing. The pH value of a solution is strongly influenced by its H⁺ ion concentration. The higher the concentration of H⁺ ions, the lower the pH value [10]. Anthocyanin pigments have higher level of stability under acidic conditions or at pH 1-4. Accordingly, anthocyanin pigment of the 80% rosella calyx extract in this research from 1 to 21 day still in normal values, which is in the range 2-3 (acid). Based on the post-hoc test results the pH value of the extract at cold temperatures more stable until 21th day of storage compared to the room temperature. Low storage temperatures can activate enzymes, maintain stability of anthocyanin pigment and delayed anthocyanin degradation [11].

Figure 2. pH value of 80% Roselle calyx extract at various temperature and days storage
The microscopic observation of nematode eggs examination was verified by 2 parasitology experts, showed score 2 (Figure 3). This means that the 80% Roselle calyx extract solution gives good staining colour of the nematode eggs samples and gives a contrast colour of the worm eggs layers. So that, this extract can be used for examination of nematode eggs for 21-day storage condition at room temperature or cold temperature.

![Image of nematode eggs with arrows indicating different stages and temperatures](image)

**Figure 3.** Microscopic observation of nematode eggs (black arrow) in various days storage (1, 7, 14, and 21 days) and various temperature (room temperature, A-D and cold temperature, E-H)

4. Conclusion

Natural extract from Roselle (*Hibiscus sabdariffa L.*) calyx stained intestinal nematode eggs and stable within 21 days storage in cold temperature (4°C).

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