

## Cytotoxic Activity of Tahongai (*Kleinhovia hospita* Linn.) Leaves Extracts Using Brine Shrimp Lethality Test

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### Abstract

Tahongai (*Kleinhovia hospita* Linn.) leaves had been known contain alkaloids, flavonoids, saponins, steroids, and tannins. At Komerang, South Sumatera province, tahongai leaves had been known to treat tumor, cancer, polyps, acne, and dysmenorrhea. The study of cytotoxic activity of tahongai bark and stem were done. This study aims to determine the cytotoxic activity of tahongai leaves extracts using BSLT method. Tahongai leaves were extracted using gradual maceration with n-hexane, ethyl acetate, and ethanol 96%. Each extract was tested cytotoxic activity towards *Artemia salina* L. larvae. The yield of n-hexane, ethyl acetate, and ethanol extracts are 2,686%, 7,033%, and 7,933% respectively. Ethanol extract of tahongai leaves had the best cytotoxic activity with lethality value 76,667% at 500ppm. Statistical analysis with two way ANOVA showed extract and concentration had a significant ( $p < 0,05$ ) effect on larvae lethality percentage.

### Keywords

*Kleinhovia hospita* Linn., *Artemia salina* L., cytotoxic, BSLT.

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## 1. INTRODUCTION

Tahongai (*Kleinhovia hospita* Linn.) is a tropical plant. Tahongai has a therapeutic effect as antidiabetic, antioxidant, hepatoprotective, anticancer, and antihyperlipidemic (Paramita, 2016). Research on cytotoxic effects of tahongai plant has been carried out by Morilla et al. (2015) using bark and stem ethanol extract with an LC<sub>50</sub> value 452,03 µg/mL. Methanol extract of tahongai leaves, stem, and bark had been studied to have cytotoxic activity in hepatoma cells and can inhibit murine leukemia cells (P388) with IC<sub>50</sub> 56 µg/mL (Nurhidayah et al., 2013). Decoct of tahongai bark and stem also have cytotoxic activity with an LC<sub>50</sub> value of 698.54 µg/mL Morilla et al. (2015).

The leaves and wood of *Kleinhovia hospita* Linn. found to contain flavonols, kaempferol, and quercetin (Arung et al., 2012). Pieme et al. (2010) found that quercetin and kaempferol had anticancer activity. Quercetin and kaempferol are one of the compounds of flavonoid that have anticancer effects both in vitro and in vivo test (Baghel et al., 2012). Based on the study above, in this study, gradual maceration was carried out to obtain active compounds from various polarity levels using n-hexane (non-polar), ethyl acetate (semi-polar) and ethanol (polar) solvents. Each extract was done to compare the cytotoxic activity using Brine Shrimp Lethality Test (BSLT).

## 2. EXPERIMENTAL SECTION

### 2.1 Materials

Plant materials used for this study comes from the same source used by Solihah et al., (2018). The chemicals used of this study were n-hexane (Brataco®), ethyl acetate (Brataco®), ethanol (Brataco®), aquadest (Brataco®), Mayer reagent (Merck®), Wagner reagent (Merck®), Dragendorff reagent (Merck®), sulfuric acid (Merck®), ammonia (Merck®), chloroform (Merck®), hydrochloric acid (Merck®), magnesium powder (Merck®), sodium hydroxide (Merck®), iron (III) chloride (Merck®), anhydrous acetic acid (Merck®), DMSO (Merck®), quercetin (FitoPure®), kaempferol (FitoPure®), and shrimp larvae *Artemia salina* Leach (Supreme®).

### 2.2 Instruments

The instruments used for this study are blender (Philips®), rotary evaporator (IKA-C Mag®), maceration chamber, water bath (Memmert™), glass tools (Pyrex®), silica TLC plate G60 F254 (Merck®), vaporizer plate (RRC®), micropipette (Dragon Lab®), vortex (Corning LSETM), 40-60 W (Itsuyama®) lighting, analytical scale of readability 0,01 and 0,0001 g (Electronic Scale® and Ohaus®), magnetic stirrer (IKA C-Mag®).

### 2.3 Extraction

A total of 1 kg dried leaves of tahongai were soaked with 6 L n-hexane where every two days the solvent was replaced until clear about ten days. Powdered pulp was macerated with 6 L ethyl acetate solvent and 6 L 96% ethanol using the same method. Filtrate evaporated using a rotary evaporator to obtain a thick extract.

### 2.4 Phytochemical Screening

#### 2.4.1 Flavonoid identification

The extracts of 0,5 g were added with 5 mL of hot ethanol for 5 minutes. The extract are filtered so that the filtrate was obtained and then a few drops of concentrated HCl are added, and 0,2 mg of magnesium powder was added. The red color indicates the presence of flavonoid group compounds.

#### 2.4.2 Saponin identification

The extracts of 1 g were added with distilled water and boiled for 2 minutes and shaken vigorously. The existence of saponins was characterized by the formation of a stable foam in a test tube.

#### 2.4.3 Alkaloids identification

The extracts of 2 g were taken then a little chloroform and sand were added and ammonia in chloroform. The mixture was shaken and then filtered. The filtrate was added with H<sub>2</sub>SO<sub>4</sub> 2N and then shaken. Two layers were formed. The top layer in the form of a water phase was separated and then tested by Mayer, Wagner, and Dragendorff reagents. The presence of alkaloids was characterized by red sediment with Wagner reagents, white sediment with Mayer reagents, and red or orange color with Dragendorff reagent (Al-Daihan and Bhat, 2012).

#### 2.4.4 Steroids and Triterpenoids identification

The bottom layer from alkaloid identification was separated and dropped onto the drop plate, allowed to dry. Anhydrous acetic acid was two drops, and one drop of concentrated sulfuric acid (Lieberman-Buchard reagent) was added to the residual. The green or red color formed indicates the presence of steroid or triterpenoid compounds (Al-Daihan and Bhat, 2012).

#### 2.4.5 Identification of Tannins

An amount of 2 g tahongai leaves extracts were added with 100 mL of water then boiled for 15 minutes then cooled and filtered so that filtrate was obtained. An amount of 1% iron (III) chloride solution was added to the filtrate. The presence of tannin compounds was characterized by the formation of dark blue or blackish green (Al-Daihan and Bhat, 2012).

### 2.5 Identification of quercetin and kaempferol using TLC

Identification of quercetin compounds on tahongai (*Kleinhovia hospita* Linn.) leaves extracts using TLC method. TLC plates were measured at 6 x 10 cm<sup>2</sup> with the upper and lower limits of 1 cm. Tahongai leaves extracts, quercetin, and kaempferol were made at a concentration of 5% and then dripped along with the silica TLC plate G<sub>60</sub> F<sub>254</sub>. The TLC plate was eluted using chloroform : ethyl acetate (5:2) eluent. Comparison of eluents

was obtained from the results of the experiment. The TLC results were aerated and detected under UV light at a wavelength of 254 and 366 nm. Then sprayed with 5% aluminum (III) chloride spray reagent in ethanol (Colegate and Russel, 1993). Results of TLC were stains or spots that glow greenish-yellow (Nuria et al., 2011).

### 2.6 Cytotoxic Activity test

#### 2.6.1 Preparation of stock solution

Each tahongai leaves extracts were dissolved in 4 drops of DMSO 10%, and 1000 ppm (mother liquor) concentration was made then a test solution with a concentration of 500; 250; 125; 62,5; and 31,25 ppm. DMSO 10% control solution was used without the addition of extracts (Morilla et al., 2015).

#### 2.6.2 Preparation of *Artemia salina* Leach Larvae

An amount of 1 L of artificial seawater (20 g of salt in 1 L of water) was needed for 1 g of *Artemia salina* Leach. eggs. *Artemia salina* Leach. egg put into a hatchery that has contained seawater or synthetic seawater then illuminated with a 40 – 60 W lamp for 24 – 48 hours. Eggs that have hatched into larvae were moved to another place, and 24 hours later, the larvae were used as test subjects (Morilla et al., 2015).

#### 2.6.3 Cytotoxic test

Six pieces of vials were used in testing each variation of the extract, which was divided into five vials for each test concentration and one vial for control. Each concentration was taken according to the calculation while for the negative control, 5 mL of seawater was added. A total of 10 larvae of *Artemia salina* Leach. The larvae put into seawater, which has been mixed with the test solution. Observations were made for 24 hours (Lisdawati et al., 2016).

### 2.7 Statistical analysis

The data of percentage of larvae lethality are expressed as the mean ± SD. Percentage of larvae lethality used as a dependent factor, whereas the type of extract and concentration used as an independent factor. The difference among the means has been analyzed by two way ANOVA.

## 3. RESULTS AND DISCUSSION

### 3.1 Extraction and phytochemical screening results

Extraction of tahongai leaves by gradual maceration method using n-hexane, ethyl acetate, and ethanol 96%. The yield of extracts can be seen in Table 1.

**Table 1.** The yield of extracts

Extract	Total (g)	% Yield
N-Hexane	26,86 g	2686%
Ethyl Acetate	70,33 g	7033%
Ethanol	79,330 g	7933%

Yield states the percentage of raw material obtained from the total raw material in the extraction process. The higher the yield value, more the opportunity for the raw material to be utilized. Percent of yield can be influenced by the duration of extraction time and the degree of fineness of the simplicia particle size. Each extract was macerated for ten days. The smaller the size of the simplicia, the higher the surface area of the particles so that the solvent is more attract compounds in the sample but if the simplicia powder is too fine, it can broken cell walls and unwanted substances (ballasts) will enter the maceration results. This is not desirable in the extraction process. The percentage of yield obtained showed that ethanol extract had the highest percentage of yield compared to other extracts of 7,933%. Ethanol as a polar solvent, which can attract polar components. This is indicates that the most polar component was found in tahongai leaves so the percentage of yield highest than another.

Phytochemical screening is an examination of the chemical content qualitatively to ensure the presence of the desired secondary metabolites contains in plant or extract. The data reveal the presence of various constituents in each extract. Phytochemical test results can be seen in Table 2.

**Table 2.** Phytochemicals screening test of tahongai leaves extracts

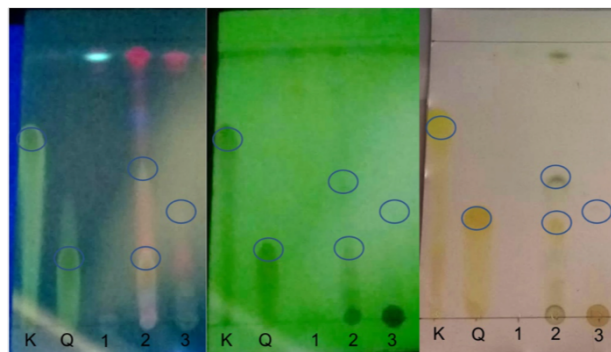
Chemical Substance	Screening Result of extracts		
	n-Hexane	Ethyl acetate	Ethanol
Alkaloid	+	+	+
Flavonoid	-	+	+
Saponin	-	+	+
Tanin	-	+	+
Triterpenoid	-	-	-
Steroid	+	+	+

All tahongai leaves extracts do not contain triterpenoids. N-hexane extract only contains alkaloid and steroid compounds. While ethyl acetate and ethanol extracts contain alkaloids, flavonoids, tannins, saponins, and steroids.

### 3.2 Identification of quercetin and kaempferol with TLC

Identification of quercetin and kaempferol in tahongai leaves extracts was used thin-layer chromatography. Stationary phase used was silica while the mobile phase used is chloroform: ethyl acetate (5:2). Spots produced by each component were observed in UV light 254 nm and UV 366 nm. UV light 254 shows that the compound has at least two conjugated double bonds while the fluorescence under UV light 365 nm shows that the component has a chromophore and has an auxochrome group in its structure (Alen et al., 2017). KLT plate was sprayed with aluminum (III) chloride ( $\text{AlCl}_3$ ) reagent. The resulting color shows a yellow color. The results showed in figure 1.

Quercetin and kaempferol compounds based on the results of TLC testing contained in ethyl acetate and ethanol extract. Quercetin and kaempferol are both polyhydroxy flavonol com-



**Figure 1.** The chromatogram of Tahongai leaves extracts (K = kaempferol; Q = Quercetin; 1 = n-hexane extract; 2 = ethyl acetate extract; 3 = ethanol extract) (a) under UV light 366 nm (b) under UV light 254 nm (c) sprayed with  $\text{AlCl}_3$

pounds. Leaves of *Kleinhovia hospita* L. had been reported contains scopoletin, kaempferol, quercetin, eleutherol and kaempferol 3-O-B-D glucoside compounds (Arung et al., 2012)

### 3.3 Cytotoxic activity

The test of cytotoxic activity of tahongai leaves extracts were used BSLT (Brine Shrimp Lethality Test) method. The cytotoxic effect of tahongai leaves extracts was determined within 24 hours after administering each extract with various concentration using *Artemia salina* as a test subject. Each test was carried out on 10 larvae then replicated 3 times. Results obtained are calculated as the percentage value.

Based on the data in table 3 it can be seen that the higher concentration of extract can cause the mortality of the larvae was greater. While the extract which has the most potential as a cytotoxic agent was the ethanol extract of tahongai leaves. In the ethanol extract of tahongai leaves contained polar compounds, such as polyhydroxy flavonoids.

The mechanism of larvae lethality of *Artemia salina* L is estimated to be related to the compounds contained in tahongai leaves extract such as flavonoids as cytotoxic agents. From the results of phytochemical screening showed that ethyl acetate and ethanol extract contained flavonoids while flavonoids were not found in n-hexane extract. Flavonoids at certain levels have the potential for acute toxicity (Carballo et al., 2002). The presence of flavonoids in the cell environment causes hydroxy groups in flavonoids to bind to cell membrane integral proteins. This causes the blocking of the active transports  $\text{Na}^+$  and  $\text{K}^+$ , the active transport stops, causing uncontrolled inclusion of  $\text{Na}^+$  ions into the cell. This causes the rupture of cell membranes (Scheuer, 2004). Rupture of cell membranes causes cell death.

Data analysis was used two way ANOVA method. Two way ANOVA was conducted to test the differences in several groups based on two independent variables. Data on percent lethality of larvae as a dependent factor, while the type and concentration of extracts as an independent factor. Data is declared homogeneous if the significance value is  $\geq 0,05$ . The homogeneity value obtained is 0,238 ( $p > 0,05$ ) so the data has the same variation.

**Table 3.** The effect of tahongai leaves extract in larvae lethality

Concentration (ppm)	Larvae lethality (mean $\pm$ SD) (%)		
	n-Hexane	Ethyl acetate	Ethanol
31,25	6,667 $\pm$ 0,577	13,333 $\pm$ 1,155	16,667 $\pm$ 0,577
62,5	13,333 $\pm$ 0,577	30,000 $\pm$ 1,000	40,000 $\pm$ 1,000
125	16,667 $\pm$ 0,577	43,333 $\pm$ 0,577	56,667 $\pm$ 0,577
250	23,333 $\pm$ 0,577	53,333 $\pm$ 0,577	70,000 $\pm$ 0,000
500	26,667 $\pm$ 0,577	66,667 $\pm$ 0,577	76,667 $\pm$ 0,577

At ANOVA values, there was type of extract variable, concentrations, and type of extract-concentration interactions. If the significance value is  $<0.05$ , the data is significant. The value obtained is a correction model of 0,000 the data is significantly different. The significance of type of extract, concentration, and type of extract-concentration interaction variables was 0,000 ( $p < 0,05$ ). This states that type of extracts, concentrations, and interactions between type of extracts and concentrations have a significant effect on percent lethality of *Artemia salina L. larvae*.

#### 4. CONCLUSIONS

Based on the results of this study, it can be concluded that the ethanol extract of tahongai leaves has the greatest yield and contain the greatest phytochemical groups compound. This correlates with its activity as a cytotoxic agent. Tahongai leaves ethanol extract has the largest cytotoxic activity compared to n-hexane and ethyl acetate extract.

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