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The Effect of Extraction Method on Total Flavonoid Content of *Ageratum conyzoides* Ethanol Extract

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ABSTRACT

This research entails a comparative study using a quantitative approach. The study focused on the Bandotan plant population in the Kudus and Jepara regions, specifically extracting leaves while excluding stems, roots, and flowers. The extraction involved maceration and soxhlet extraction using a 96% ethanol solvent. To ascertain the total flavonoid content of *Ageratum conyzoides* leaf extraction, the UV Vis Spectrophotometric method was employed. Results revealed that *Ageratum conyzoides* leaf extract obtained through the Soxhlet extraction method exhibited a higher total flavonoid content (0.32% b/v) compared to the Maceration method (0.22% b/v). The *Mann Whitney U Tes*t was conducted to analyze the total flavonoid test results. The test yielded an Asymp. Sig. (2-tailed) value of 0.102 > 0.05. Consequently, it was concluded that there is no significant difference between the maceration and soxhlet extraction extraction methods in terms of total flavonoids in *Ageratum conyzoides* extract. Despite the lack of statistical significance, it's noteworthy that the percentage of total flavonoids extracted via the soxhlet extraction method was higher than that of the maceration method.

Keywords: extraction method, total flavonoids, *Ageratum conyzoides.*

INTRODUCTION

Extract is a liquid preparation made by extracting plant material, which is soaked using a non-water (non-polar) or half-water solvent, such as dilute ethanol, for a certain period of time in accordance with the rules in the official pharmaceutical book (Departemen Kesehatan RI, 2000). Meanwhile, extraction is the process of separating and isolating substances from a substance with the addition of a certain solvent to remove components of a mixture of solid or liquid substances (Rezki et al., 2015). Another definition of extraction is the separation of active substances from a solid or liquid using a solvent (Prayudo et al., 2015). Therefore, the definition of extraction in this study is a process of separating one or more components from a homogeneous mixture using a liquid solvent as a separating agent using a certain method.

Extraction methods of natural materials are divided into cold extraction methods

and hot extraction methods. Cold extraction in principle does not require heating. This method means that there is no heating process during the extraction process, the goal is to avoid damage to the compound (Andriani, 2018). Cold extraction is used for natural materials that contain chemical components that are not resistant to heating and natural materials that have a soft texture, such as leaves and flowers (Kiswandono, 2017). An example of cold extraction is maceration. Maceration is the process of soaking the sample to attract the desired components under cold conditions (A. A. B. Putra et al., 2002). The advantage of the maceration extraction method is that the natural ingredients do not become decomposed because the procedures and equipment used are simple and not heated. Cold extraction allows many compounds to be extracted, although some compounds have limited solubility in solvents at room temperature (Puspitasari & Prayogo, 2017).

Maceration, a straightforward extraction method, derives its name from the Latin word 'macere' meaning to soak. This process involves finely grinding the simplicia, followed by soaking it in a solvent. Through this method, the solvent permeates and softens the cell structure, enabling the dissolution of soluble substances and the extraction of desired compounds. The principle of maceration is the extraction of active substances by soaking the powder in a suitable solvent for several days at room temperature protected from light (Tantrayana & Zubaidah, 2015), the solvent will enter the plant cells through the cell wall. The contents of the cell will dissolve because of the difference in concentration between the solution inside the cell and outside the cell. High concentration solution will be pressed out and replaced by a solve with low concentration (diffusion process). These events will repeat until there is a balance between the solution inside the cell and the solution outside the cell.

Maceration is typically conducted at a temperature ranging from 15°C to 20°C for a duration of 3 to 7 days to extract substances from the plant material. Generally, the process involves combining 10 parts of finely ground simplicia with a suitable degree of fineness into a vessel and then adding 75 parts of the liquid. The vessel is sealed, and the mixture is left for 5 days, shielded from light while being periodically stirred. After 5 days, the pulp is strained, and enough distillation liquid is added to the pulp, ensuring a thorough blend to extract the entirety of the juice, resulting in a total of 100 parts. The container is sealed and kept in a cool, dark place for 2 days, following which the sediment is separated (Fransisca, 2017).

The Indonesian Pharmacopoeia outlines specific requirements for the distillate

used, which includes water, ethanol, ethanol-water, or ether. Ethanol serves as a recommended distillate due to several advantageous properties. It demonstrates selectivity in extraction processes, making it challenging for molds and germs to thrive in concentrations of 20% or higher. Ethanol is non-toxic, neutral, and possesses excellent absorption qualities. Additionally, it seamlessly mixes with water at any ratio and requires less heat for concentration. Ethanol's solubility properties allow it to dissolve various compounds such as alkaloid bases, volatile oils, glycosides, curcumin, coumarin, anthracinone, flavonoids, steroids, and chlorophyll. However, it only exhibits limited solubility for lipids, tannins, and saponins. Consequently, ethanol aids in restricting the presence of interfering substances in the distillate. (Departemen Kesehatan RI, 2020).

Furthermore, the hot extraction method is an extraction method that uses heating in extracting simplicia with less solvent and the time used is faster. One example of a hot extraction method is soxhlet extraction (Putra A. A. G. R. Y., 2016). Soxhlet extraction is a method of separating components contained in solid samples by repeated extraction with the same solvent, so that all the desired components in the sample are perfectly isolated (Ridwan et al., 2017). The advantages of the soxhlet extraction method are that the solvent used is relatively less, the time used is faster, can produce more extracts and the sample can be extracted perfectly because it is done repeatedly (Tapalina et al., 2022).

Unlike maceration, soxhlet extraction involves the repeated filtration of a component present in a solid substance using a specific solvent, aiming to isolate all desired components. This extraction method employs a specialized apparatus known as a soxhlet extractor. Compared to maceration, soxhlet extraction typically yields higher quantities of extracted substances (Anam et al., 2014). The soxhlet extraction method is effective for isolating compounds with limited solubility in a solvent, as well as impurities that do not dissolve in the solvent. This method specifically targets solid samples, earning it the moniker 'solid-liquid extraction' (Melwita et al., 2014).

The solvent selected for the soxhlet extraction method needs to meet specific criteria, including: (1) volatility, (2) a low boiling point, (3) the ability to dissolve the sample compounds, (4) easy separation when agitated within a short duration, and (5) compatibility with the nature of the compounds to be isolated (polar or nonpolar) (Febryanto, 2017). Soxhlet extraction typically operates within a short duration, occurring at the boiling point of the solvent (Harfayati et al., 2020).

The soxhlet extraction process operates based on the heating of the distiller liquid within the flask, generating steam. The heated liquid vapor ascends through the side pipe and undergoes condensation within the condenser. The condensed liquid then drips through the tube containing the simplicia powder, returning to the flask (Departemen Kesehatan RI, 2000). The essential tools employed in soxhlet extraction include the soxhlet apparatus, round bottom flask, heater, and condenser. The soxhlet device comprises a siphon, F pipe, and lead. Halting the ongoing heating stops the soxhlet extraction process (Firyanto et al., 2020). Soxhlet extraction offers several advantages over other methods. It ensures repeated contact between the sample and pure solvents, yielding larger sample extractions regardless of solvent volume and requiring less time. However, a drawback of soxhlet extraction is that the continuous heating during the extraction process can potentially damage other components (solutes) within the sample compound (Febryanto, 2017).

Ageratum conyzoides Linn also known as Bandotan has been included in the *Global Invasive Species Database* (GISD, 2022), because it is one of the invasive plant species, but empirically many studies have been conducted related to the benefits of Bandotan plants for wound healing and health. *Ageratum conyzoides* is an annual herbaceous plant that can grow up to 1 m in height. The stems and leaves of the plant are covered with fine white hairs. The plant has many beneficial effects in medicine and can be used in the search for new drugs from herbs. This the reason why we focused on this plant. In Indonesia, *Ageratum conyzoides* plants are only used for animal feed and have not been utilized properly as herbal plants. This is because not many people know the antioxidant content contained in this plant.

The leaves and roots of the plant are known to contain alkaloids, flavonoids, tannins, saponins, cardiac glycosides and anthraquinones, minerals, vitamins and other compounds that have pharmacological activity. In leaves, phytochemical compounds with high concentrations are alkaloids, flavonoids and tannins (Melisa, 2020). Flavonoids are secondary metabolites of polyphenols, found widely in plants and foods and have various bioactive effects including anti-viral, anti- inflammatory (Wang et al., 2016).

Flavonoids include natural phenolic compounds that have potential as antioxidants and have medicinal bioactivity. In addition, flavonoids function as antioxidants in the human body so they are very good for cancer prevention (Nisa Kasmui, 2015). The benefits of flavonoids include protecting cell structure, increasing

the effectiveness of vitamin C, anti- inflammatory, preventing bone loss and as antibiotics (Waji & Sugrani, 2009). Based on the differences in the two extraction methods and the potential of *Ageratum conyzoides* leaves, it is important to know the differences in the total flavonoid content in *Ageratum conyzoides* leaf extracts. Therefore, this study aims to determine the effect of extraction methods (maceration and soxhlet extraction) on the total flavonoid content of *Ageratum conyzoides* extract.

METHOD

This research is a type of comparative research using a quantitative approach. The study population was the population of Bandotan plants in the Kudus and Jepara regions. Meanwhile, samples were taken using *simple random sampling* technique. Bandotan plant samples were taken from Kudus (Bae, Jati, Mejobo, Gebog sub-districts), and Jepara (Pecangaan, Nalumsari sub-districts). The organ parts taken from the plants were leaves, while the stems, roots and flowers were not extracted in this study.

Furthermore, to determine the flavonoid content of the extraction results of *Ageratum conyzoides* leaves, it was carried out using the UV Vis Spectrophotometry method. Standard Curve Preparation Procedure is carried out as follows:

1. Weigh carefully the Quercetin standard as 10.0 mg
2. Add with 0.3 mL of 5% sodium nitrite, wait 5 min.
3. Add with 0.6 mL of 10% aluminum nitrate, wait 5 minutes.
4. Add with 2 mL of 1 M sodium hydroxide.
5. Adjust to a volume of 10 mL
6. Dilute to standard curve concentration
7. Absorbance read at λ 510 nm

The procedure for determining the total flavonoids equivalent of Quercetin by spectrophotometric method is as follows:

1. Take a sample of 0.2 mL, evaporate.
2. Add with 0.3 mL of 5% sodium nitrite, wait 5 min.
3. Add with 0.6 mL of 10% aluminum nitrate, wait 5 minutes.

4. Add with 2 mL of 1 M sodium hydroxide.
5. Precise to a volume of 10 mL, dilute 10 times
6. Read the absorbance at λ 510 nm.

Table 1. Dilution (1000 ppm standard solution)

Conc	*0.5*	*1*	*2*	*5*	*10*	*25*	*50*	*75*	*100*	*ppm*
Lart Induk	5	10	20	50	100	250	500	750	1000	µl
Aquabides	9995	9990	9980	9950	9900	9750	9500	9250	9000	µl
Volume	10	10	10	10	10	10	10	10	10	ml

The results of the total flavonoid test were then analyzed using the *Mann Whitney U Test*, to determine if the difference between the two extraction samples is significant.

RESULT AND DISCUSSION

The active compounds contained in Bandotan are flavonoids, alkaloids, chromenes, benzofurans, and pentenoids. The highest content of active compounds in bandotan is flavonoids, especially quercetin. Quercetin is a flavonol compound, has antioxidant activity and is widely explored for therapeutic use (Sutjiatmo et al., 2020). Based on this, it is important to determine the total flavonoid content in Bandotan plants with different extraction methods.

Prior to the extraction of Bandotan leaves, researchers made Bandotan leaf simplicia. The simplification was carried out as follows:

1. Picking Bandotan leaves (in this study Bandotan leaves were taken from the Kudus and Jepara areas).
2. Clean Bandotan leaves with running water,
3. Drying Bandotan leaves in the sun (2-3 days),
4. Puree the dried Bandotan leaves using a blender/sharpener,
5. Sieve the Bandotan leaves that have been finely ground, until a simplicia of almost the same size is obtained.

The preparation of Bandotan leaf extract in this study used 2 extraction methods, namely the Maceration method and the Soxhlet extraction method with 96% ethanol as solvent. This aims to determine the difference in total flavonoids contained in each extract. This is appropriate based on several relevant research studies, *Ageratum conyzoides* leaf extract is obtained through the maceration method using 96% ethanol (Rianosa et al., 2020). The results of other studies show that leaves from ethanol extracts have higher antioxidant activity compared to extraction using water/ distilled water (Kotta et al., 2020).

1. Maceration Method

In the first maceration method, using a mass of simplicia as much as 20 grams with a volume of ethanol 200 ml (ratio 1: 10). Duration of soaking time is 7 days. Furthermore, the filtering and distillation process is carried out, to remove ethanol in the solution. The second and third maceration (remaceration) was carried out using simplicia from the previous maceration, filtering and simple distillation were carried out. The results of the first maceration and remaceration obtained a thick extract, then as much as 5 ml of the extraction results continued to test the total flavonoid content.

2. Soxhlet extraction Method

Extraction using the Soxhlet extraction method was carried out at the Chemistry Laboratory of the Faculty of Tarbiyah IAIN Kudus. The mass of simplicia used was 20 grams with a volume of ethanol Soxhlet extraction as much as 235 ml. Soxhlet extraction consisted of 6 cycles, with the following time records:

a. Cycle 1 = 11:20 - 12:04 with a time length of 44 minutes
b. Cycle 2 = 12.04 - 12.36 with a time length of 32 minutes
c. Cycle 3 = 12.36 - 13.00 with a length of 24 minutes.
d. Cycle 4 = 13.00 - 13.25 with a length of 25 minutes
e. Cycle 5 = 13:25 - 13:45 with a duration of 20 minutes
f. Cycle 6 = 13.45 - 14.09 with a length of 24 minutes.

The distillation process is then carried out and a thick extract of Bandotan leaves is produced. A total of 5 ml from the extraction results will be continued for spectrophotometric tests to determine the total flavonoid content. Data from the total flavonoid content test can be seen in Table 2 below.

Table 2. Total flavonoid content of Quercetin Equivalent Spectrophotometric

Sample results extract	***Sample volume (mL)***	***Final add (mL)***	***FP***	***Tool results (ppm)***	***Acquisition results (% b/v)***	***Average (%b/v)***
Maceration	0,200	10	10	4,5976	0,23	**0,22**
	0,200	10	10	4,3378	0,22	
Soxhlet extraction	0,200	10	10	6,8766	0,34	**0,34**
	0,200	10	10	6,7753	0,34	

Based on the test results using the spectrophotometric method in Table 2, it can be seen that *Ageratum* leaf extract using the Soxhlet extraction method has a higher total flavonoid content (0.34% b/v) compared to Ageratum leaf extract using the Maceration method which is 0.22% b/v. However, to find out the significant difference between the two extraction results, it is necessary to test using the *Mann Whitney U Test*.

Table 3. *Mann Whitney U Test* results.

Statistical Test	Total flavonoids
Mann-Whitney U	0.000
Z	-1.633
Asymp. Sig. (2-tailed)	0.102
Exact Sig. [2*(1-tailed Sig.)]	0.333

Based on the *Mann Whitney U Test* results in Table 3, it shows that the Asymp. Sig. (2-tailed) of 0.102, the value is greater than the significance value of 0.05. Therefore, it can be concluded that Ho is accepted, so it can be said that there is no difference between maceration and soxhlet extraction extraction methods on the total flavonoids of *Ageratum conyzoides* extract.

The results obtained in this study are in accordance with several relevant research studies, namely quantitatively the percentage of total flavonoids with the soxhlet extraction method has a higher flavonoid content compared to the maceration method. However, it did not end there, this study showed that the difference in total flavonoid concentration from the two extraction methods was not significant. The higher total flavonoids from the Soxhlet extraction method can be caused by the more

cycles performed in the Soxhlet extraction method. The more cycles performed, the more the total flavonoid content will be produced, because the temperature is higher.

In general, the solubility of the extracted active substance will increase with increasing temperature. However, increasing the extraction temperature also needs to be considered, because too high a temperature can cause damage to the material being processed (Margaretta et al., 2011). Extraction time is also very influential on the compounds produced. According to Budiyanto et al. (2008), the right extraction time will produce optimal compounds. Extraction time that is too long will cause the extract to hydrolyze, while extraction time that is too short causes not all active compounds to be extracted from the material.

Purwanti (2022) states that Bandotan leaves (*Ageratum conyzoides* L.) are often called nuisance plants. Bandotan grows wild in yards, plantations and fields. The results of phytochemical screening showed that Bandotan extract contained alkaloids, flavonoids, tannins, saponins, and steroids. The results of photometric analysis showed that the average concentration of flavonoids and tannins in Bandotan extracts was higher than that in the other extracts. The extracts of bandotan leaves from soxhlet extraction were 177.98 mg/g and 61.13 mg/g, higher than those from maceration which were 133.67 mg/g and 47.75 mg/g. This shows that the extraction method affects the concentration of chemical compounds in the extract.

Total flavonoid levels in *Ageratum conyzoides are* not only influenced by the method, temperature, extraction time, but also influenced by the type of terrain where the plant lives. Yuliani et al. (2019) stated that the total flavonoid content in *Ageratum conyzoides* in the highlands (3.2 ± 0.06 mg/mL) was higher than *Ageratum conyzoides* growing in the middle plains (2.9 ± 0.0 mg/mL) and in the lowlands (2.6 ± 0.06 mg/mL).

The type of flavonoids contained in *Ageratum conyzoides* consists of five methoxylated flavonoids which were isolated and identified based on mass by NMR spectroscopic analysis namely (1) 5,6,7,8,5′-pentamethoxy-3′,4′-methylenedioxyflavone (eupalestin); (2) 5,6,7,5′-tetramethoxy-3′,4′-methylenedioxyflavone; (3) 5,6,7,8,3′,4′,5′-heptamethoxyflavone (5′-methoxynobiletine); (4) 5,6,7,3′,4′,5′-hexamethoxyflavone and (5) 4′-hydroxy-5,6,7,3′,5′-pentamethoxyflavone (ageconyflavone C) (Nour et al., 2010).

Mutingatun et al. (2022) explained that the results of phytochemical screening tests showed that leaf powder and ethanol extracts of bandotan leaves contained alkaloids, flavonoids, saponins, tannins, quinones, steroids, and triterpenoids. The total flavonoid content of ethanol extract of bandotan leaves amounted to 129.27 mg/g extract. Identification of flavonoid isolate using UV-Vis spectrophotometer showed that flavonoid isolate belongs to flavanone compound group with maximum absorption at wavelength of 315 nm (band I) and 280 nm (band II). FTIR analysis showed that the flavonoid isolate has OH, CH aromatic, CH alkane, C=O, C=C aromatic, CO ether, CO alcohol, and substituted aromatic ring functional groups. Identification of flavonoid isolate structure by spectrophotometry and addition of shear reagent and FTIR, it is suspected that the isolate is a 4'-hydroxy flavanone compound.

Based on this discussion, the total flavonoid content in the ethanol extract of Bandotan leaves shows a difference in concentration between the maceration and soxhlet extraction method but not significant. This means that to obtain the total flavonoid content, we can use hot or cold extraction methods, if using hot extraction methods such as soxhlet extraction we need to pay attention to the optimal temperature and extraction time used. In this study, the soxhlet extraction method carried out a maximum of 6 cycles with a time of 169 minutes or 2 hours 49 minutes.

CONCLUSION

Analysis via the UV-Vis spectrophotometry method revealed that *Ageratum conyzoides* leaf extract obtained through the soxhlet extraction method exhibited a higher total flavonoid content (0.32% b/v) compared to Bandotan leaf extract obtained via the maceration method, which measured 0.22% b/v. The results of the *Mann Whitney U Test* indicated an Asymp. Sig. (2-tailed) value of 0.102, surpassing the significance threshold of 0.05. Thus, it can be concluded that there's no significant difference between the maceration and soxhlet extraction methods concerning the total flavonoids in *Ageratum conyzoides* extract.

While the comparison yielded no statistically significant difference between the two extraction methods in terms of total flavonoids in *Ageratum conyzoides* extract, the soxhlet extraction method exhibited a higher percentage of total flavonoids compared to maceration. This increase in total flavonoids via the soxhlet extraction method might be attributed to the multiple cycles involved in this extraction technique. The greater

number of cycles tends to enhance the total flavonoid content, as temperature significantly influences flavonoid production. When utilizing hot extraction methods like soxhlet extraction, optimizing temperature and extraction duration is crucial for achieving the desired results.

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