

# Influence of Extraction Parameters on the Yield, Phytochemical, TLC-Densitometric Quantification of Quercetin, and LC-MS Profile, and how to Standardize Different Batches for Long Term from *Ageratum conyzoides* L. Leaves

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## ABSTRACT

**Introduction:** *Ageratum conyzoides* Linn is one of the Indonesian herbs are used as folk medicine. There are many studies to the therapeutic activity. However, the study of the influence in extraction parameters and how to standardize different batches of plant is still limited. **Objective:** The present study was designed to investigate the influence of extraction parameters on the variables matter, yield, phytochemical profile, and total quercetin from *A. conyzoides* L. leaves extract. The study also evaluates Liquid Chromatography-Mass Spectroscopy (LC-MS) and TLC fingerprint profiles of the extract to determine the proper quality of plant material of *A. conyzoides* L. leaves. **Methods:** Different temperatures (from 0°C to 60°C), solvent types (water and 70% ethanol (%v/v)), and extraction time (192 h VS 2 h) were used to investigate the influence of extraction parameters. The profiles of extract were performed by TLC and LC-MS method. **Results:** The extract yield was influenced by temperature, solvent types, and time extraction. Our results suggest that the digestion technique at 60°C with 70% ethanol for 2h was recommended for the extraction method to obtained high yield and quercetin content as bioactive compound in *A. conyzoides* L. leaves extract. The range of quercetin content (40–52 ppm) combined with TLC and LC-MS profile can be used to determine the proper quality of plant material. **Conclusion:** All parameter of extraction have an affect on the yield extract and total quercetin on the final extract. Thus, there is a need an optimized method to obtain the suitable method.

**Key word:** *Ageratum conyzoides*, TLC-Densitometric, Quercetin, LC-MS, Standardization.

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## INTRODUCTION

*Ageratum conyzoides* Linn. also known as Bandotan or Babandotan, is one of the Indonesian herbs are used as folk medicine for treating disease include wound dressing, skin diseases, ophthalmic, colic, ulcers treatment, diarrhoea, dysentery, fever, anti-inflammatory, etc.<sup>1</sup> Many studies have proven the activity of *A. conyzoides* L. protect against anxyolitic,<sup>2</sup> pediculosis,<sup>3</sup> damages generated from arsenic exposure,<sup>4</sup>etc. The activity of the metabolites in biological conditions might be due to by the presence of secondary metabolites or phytochemical, including alkaloids, flavonoids, chromenes, benzofurans, and terpenoids.<sup>5</sup> Flavonoids, especially quercetin content is the mostly explored phytochemicals for therapeutic potential in *A. conyzoides* L. For the example, Awad *et al* reported that quercetin from the ethyl acetate extract of *A. conyzoides* L. leaves have anti-inflammatory activities against carrageenan induced edema.<sup>6</sup> Hence, the present study focused into quercetin to evaluate the influence of extraction parameters on the responsible variables extractable matter (yield, extract profile, and quercetin content).

We have known since decades that chemical constituents as extractable matter which obtained from the extraction process were influenced by extraction parameters, also influenced by quality of medicinal plant, including an environmental factors, method of cultivation, time of collection, post-harvest, processing.<sup>7</sup> Thereby, there is need a tool to evaluation the chemical profile and quantification of bio-active content to standardize different batches of plant material to ensure the reproducibility quality of herbal medicinal product.<sup>8</sup> Therefore, this study also investigates the chemical profile using TLC and LC-MS method. The extractable matter, TLC<sup>9</sup> and LC-MS<sup>10</sup> fingerprint can be used as a reference for standardization of quality of herbal medicines.<sup>9,10</sup>

## MATERIALS AND METHODS

### Chemical

Quercetin (powder) as reference standard was purchased from Sigma-Aldrich (Germany). Water,

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methanol, and acetonitrile for chromatography (LC-MS Grade) LiChrosolv® were purchased from Merck (Darmstadt, Germany). All other chemicals for analysis, including ethanol, ethyl acetate, toluene, formic acid for analysis (Emsure® Grade) were purchased from Merck (Darmstadt, Germany).

#### Collection and authentication of plant material

The plant material of *A. conyzoides* leaves were collected from three different places in Java, among others Indonesian Spice and Medicinal Crops Research Institut (ISMCR) (West Java), Purwokerto (Central Java), and Materia Medica (East Java). *A. conyzoides* L. leaves were identified by Indonesian Institute of Sciences, Research Center for Biology with identification number 1626/IPH.1.01/If.07/VII/2016. The leaves were separated, washed, dried, milled into powder, and then stored in a closed container for further use.

#### Preparation of extracts using maceration technique

One hundred grams (100 g) of *A. conyzoides* L. leaves powder was extracted using ethanol (70%, %v/v) at room temperature for 192 hours. After 192h, the mixtures were filtered with vacuum filtered using Buchner funnel. After that, the filtrate was evaporated at 60°C under reduced pressure using Buchi Evaporators R-215 (integrated vacuum controller, water bath, and diagonal V-condenser, Switzerland) to obtain the concentrated extract. Then, the concentrated extract was dried in the oven Memmert. Dried extract stored in the closed glass bottle for further use, namely crude ethanolic extract. The crude water was prepared by the same method of ethanolic crude extract, but crude water was made in the pure water.

#### Preparation of extracts using digestion technique

One hundred grams (100 g) of *A. conyzoides* L. leaves powder was extracted using ethanol (70%, %v/v) at 40, 50, 60, and 70°C, respectively for 2h. After 2h, the mixtures were filtered with vacuum filtered using Buchner funnel. After that, the filtrate was evaporated at 60°C under reduced pressure using Buchi Evaporators R-215 (integrated vacuum controller, water bath, and diagonal V-condenser, Switzerland) to obtain the concentrated extract. Then, the concentrated extract was dried in the oven Memmert. Dried extract stored in the closed glass bottle for further use, namely crude ethanolic-D extract. The crude water was prepared by the same method of ethanolic crude extract, but crude water was made in the pure water.

#### Preparation of sample solution

Each sample of dried *A. conyzoides* L. leaves extract (2 mg) was dissolved in 70% ethanol (1 mL). All solutions were filtered through a 0.45 µm nylon membrane filter (Whatman). The sample was filtered before spotting.

#### Preparation of standard solution

Quercetin reference standard (Sigma-Aldrich, Germany) was accurately weighed and dissolved in 70% ethanol for the preparation of stock solution (200 ppm). Standard working solution of quercetin was prepared by diluting the stock solution with ethanol 70% in a 10 mL volumetric flask to obtain working standard solution at the concentration of 20, 40, 60, 80, 100, and 120 ppm.

#### Thin layer chromatography study for phytochemical profiling

Each sample solution and standard were spotted by automatic sampler (Automatic TLC Sampler 4, CAMAG) on a 60 F254 silica gel plate (Merck, Germany) and subsequently eluted using solvent system of toluene, ethyl acetate, and formic acid in ratio of 58: 33: 9 (v/v/v) with track distance 0.50 cm and the migration distance was 90 mm. Detection was done under ultraviolet at 254 and 366 nm without chemical treatment. In addition, all spots clearly visible at white light and 366 nm after

derivatized with aluminium chloride (AlCl<sub>3</sub>) 1% (%w/v in ethanol) and sulphuric acid (H<sub>2</sub>SO<sub>4</sub>) 10% (%v/v in water). CAMAG TLC Visualizer with winCATS software was used for imaging, analysis, and documentation of TLC.

#### Thin layer chromatography study for quantification of quercetin

Each sample solution and all of each standard solution were spotted on a 60 F254 silica gel plate using automatic sampler (Automatic TLC Sampler 4, CAMAG) and subsequently eluted using solvent system of toluene, ethyl acetate, and formic acid (58:33:9) with track distance 0.50 cm and the migration distance was 90 mm. Detection was done under ultraviolet at 254 and 366 nm without chemical treatment. In addition, all spots clearly visible at white light and 366 nm after derivatized with aluminium chloride (AlCl<sub>3</sub>) 1% (%w/v in ethanol) and sulphuric acid (H<sub>2</sub>SO<sub>4</sub>) 10% (%v/v in water). The fluorescence emission of quercetin (R<sub>F</sub> = 0.51) was recorded under UV radiation at 366 nm, by means of a digital camera, the images were analysed by CAMAG TLC Scanner 3 with winCATS software. The amounts of quercetin in the samples were calculated by comparing densitogram peak areas from the samples with those from the standards on the same plate at maximum wave length (425 nm). For determination of calibration equations 20, 40, 60, 80, 100, and 120 ppm for quercetin standard were chromatographed.

#### LC-MS Analysis for secondary phytochemical profiling

The sample extract (5 mg) was dissolved in 1 mL methanol (LC-MS grade, Chromasolv) and filtered with Whatman membran filters nylon pore size 0.2 µm (Sigma-Aldrich). Then, the extract (5 µL) was analysed on an UPLC-QtoF-MS/MS System (Waters Corporation, Milford, MA). Sample extract (5 µL) was separated on an ACQUITY UPLC BEH C18 Column (1.7 µm × 2.1 mm × 50 mm) at 40°C. A gradient elution method was used with water-formic acid 0.1% (%v/v) as solvent A and acetonitrile-formic acid 0.1% (%v/v) as solvent B, flowing 0.3 mL/min. It started with A:B in ratio 95:5 for the first minutes, then increased the linearity with solvent B for 6 min, and hold at solvent with A:B in ratio 95:5 from 7.5 min to 9 min. The total chromatographic run time was 9 min. High definition mass spectrometry was performed on a XEVO-G2-Quadrupole (Q)-Time of Flight Mass (ToF) system (Waters, Milford, MA) in V-optics and operated in Electrospray Ionization (ESI) positive (resolution mode) to enable detection of secondary metabolite compounds. The optimal conditions of analysis were as follows: Capillary voltage of 3 kV, the sampling cone at 38 V, desolvation temperature at 300 °C, source temperature at 110 °C, flow rate of desolvation gas at 500 liters/ hours, and flow rate of cone gas at 16 liters/ hours.

## RESULTS AND DISCUSSION

#### The effect of extraction conditions on yield

There are many steps to obtain the phytochemicals from plant, such as milling, grinding, homogenization, and extraction.<sup>11</sup> The extraction process is the important step to minimize interference from compounds that may co-extract with the target compound, so we can obtain the extract with high content of bio-active compound.<sup>12</sup> It is believed that bioactive compounds from plant foods may have health beneficial effects and reduce the risk of diseases.<sup>13</sup> This work focused on the discussion of effect of various extraction conditions to the chemical compounds and quercetin content in the extract as extractable matter. The extractable water can be used as indicator to choose the suitable extraction technique. These results are shown in Figure 1.

As shown in Figure 1, the extraction time showing a significant effect on total yield extract from *A. conyzoides* L. Based on the results, the highest yield extract was obtained using water as solvent. Based on the results, the highest extract yield obtained by crude water extract (30.20% w/w), followed by crude ethanolic extract (15% w/w) when the

process of extraction using maceration technique for 192h. These results are in accordance with the expected that a longer extraction time generally led to a higher percentage yield. The longer extraction time values allow for the longer amount of time the solute and solvent were in contact, so it make the systems have been successfully mass transfer. However, the long of extraction time may cause the additional cost.

As described in Figure 1, heat-extraction methods shown to have advantages of savings in time and energy. As shown in Figure 1, digestion technique at 60 °C only for 2h using water as a solvent can produce the yield extract as much as 14.10% (%w/w). It indicates that the digestion method had a positive effect to reach an optimum yield extract with minimum extraction time.

Figure 1 gives us information about that the aqueous solvent had a positive effect to reach an optimum yield extract. For the example, the extraction yield of water extract from digestion method for 2h at 40°C is 12.90% (%w/w), while the ethanolic extract only 8.90% (%w/w). This may be attributable to higher solubility of sugars and oligosaccharides in water than ethanol, so the yield of water is higher than ethanol. These results seem to be in line with the effect of various solvent systems on extraction sugar from beach pea (*Lathyrus maritimus* L.)<sup>14</sup> and some medicinal plants.<sup>15</sup>

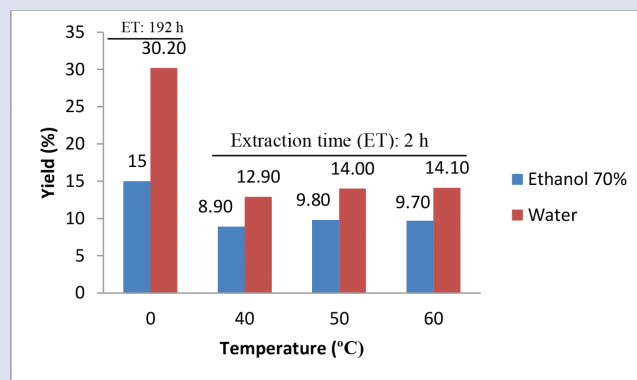
#### Phytochemical profile of *A. conyzoides* L. leaves extracted via different extraction methods.

An extraction technique of medicinal plants contributes significantly to the final quality of the herbal drug.<sup>16</sup> For the example, temperature's effect on extraction is dual. On one hand, higher heating process can destroy certain chemical compound in the extract and may adversely affect the efficacy of medicinal plants,<sup>17</sup> thereby there is need a tool to ensure the quality of the extract. In the last 20 years or so, an online chromatographic system has emerged as the most important method for the identification of target and unknown chemical compounds.<sup>18</sup> Therefore, the present study was used TLC to evaluate the chromatographic pattern of the *A. conyzoides* L. leaves extract and the result shown in Figure 2.

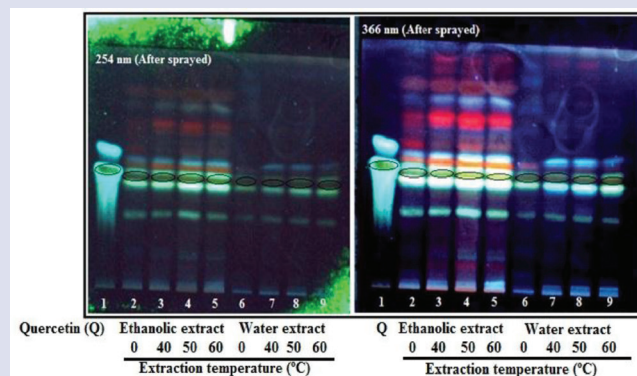
Chromatographic patterns for ethanolic extract were shown on track 2 to track 5, while the patterns for water extract were shown on track 6 to track 9. As shown in Figure 2, chromatographic patterns of all samples were separated well using toluene: ethyl acetate: formic acid in a 58: 33: 9 ratios. Furthermore, the spot of all samples were seen clearly under ultraviolet (UV) light at 245 nm and 366 nm after sprayed with aluminum chloride (AlCl<sub>3</sub>) 1% (%w/v in ethanol) and sulfuric acid (H<sub>2</sub>SO<sub>4</sub>) 10% (%v/v in water). Based on Figure 2, the chromatographic pattern of all samples of ethanolic extract (track 2 to track 5) were shown almost have the same pattern. This also occurs in chromatographic patterns of water extract (track 6 to track 9). These results were indicated that the heat process in extraction process (track 3, 4, 5 for ethanolic extract and track 7, 8, 9 for water extract) did not damage the compounds in extract.

Intermolecular attraction between the eluent and the compounds determine the solubility of the mobile phase. The polarity of the mobile phase was used in this experiment is semi polar. As shown in Figure 2, many secondary metabolites in ethanolic extract more attracted in the mobile phase, while the compounds in the water extract spends more time in adsorbent (polar). In addition, Figure 2 showed that many compounds as shown as spots more attracted with eluent than with adsorbent. It indicates that, many compounds in *A. conyzoides* L. leaves are semipolar.

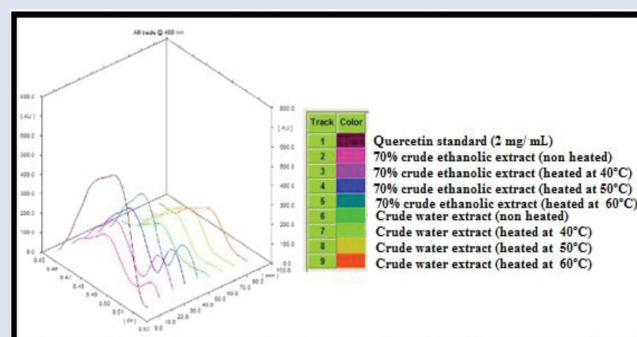
As shown in Figure 2, the chromatographic patterns of ethanolic extract have same profile with the chromatographic pattern in water extract, especially spot at R<sub>f</sub> 0.51 which marked by black circle spot. Moreover, the sample of sample extract at R<sub>f</sub> 0.51 equal to the spot of standard quercetin. These results indicate that quercetin compounds have been extracted successfully from the leaves of *A. conyzoides* L. The identity



**Figure 1:** Effect solvent, extraction time, and temperature on total yield extract



**Figure 2:** TLC profile of *A. conyzoides* L. leaves extract at 254 and 366 nm (after sprayed with aluminium chloride 1% and sulfuric acid 10%) which obtained by different extraction methods. Extraction time in maceration technique (0°C) is 192h, while extraction time in digestion technique is only 2h



**Figure 3:** Spectra overlay of quercetin corresponding peak in all extracts of *A. conyzoides* L.

of the quercetin spots in the sample was confirmed by comparing the UV-Vis absorption spectra between spectra of samples and standard using a CAMAG TLC Scanner 3 (Figure 3).



### The effect of extraction conditions on total quercetin content

The previous study reported that flavonoids and tannin content from the ethanolic extract of the leaves of *Ageratum conyzoides* L. has anti-inflammatory activity.<sup>19</sup> Awad *et al* in 2013 reported that quercetin isolated from the mucilage of crude extract of *A. conyzoides* L. leaves has anti-inflammatory activities against carrageenan induced edema.<sup>6</sup> Therefore, the present study was used quercetin content to evaluate the influence of extraction conditions and also as a guide to choose a suitable extraction technique for *A. conyzoides* L. which can produce high of quercetin content. The content of quercetin was determined using TLC densitometric method and the results are shown in Figure 4.

As shown in Figure 4, total quercetin in crude ethanol higher than in crude water. This may possible by phenolic compounds including quercetin is more attracted in ethanol than in water. It may caused by interaction between hydrogen in phenol compounds and polarity of solvent. The increasing of carbon chain length of solvent will increase the solubility of hydrogen of phenols compounds in alcohol.<sup>20</sup>

Figure 4 was confirmed that temperature parameters varied in this study affected the quercetin content in *A. conyzoides* L. leaves extract. Figure 4 showed that, the quercetin content in the *A. conyzoides* L. leaves extract can enhanced with increasing temperature up to 60 °C in various solvent, although the time of extraction is only 2h. Thus, these results indicated that the temperature during extraction process have influence against quercetin content. The increase in quercetin accumulation is may related to the Maillard reaction products which have responsibility for the increase in quercetin content as a natural phenolic compound after heating. The Maillard reaction can liberate the cleaving of the esterified and glycosylated bond in phenolic compounds.<sup>21</sup>

Figure 4 showed that the longer extraction time only did not increase the quercetin content in the extract. Total quercetin content in sample extract which obtained from maceration technique lower than sample extract from digestion technique. For the example, total quercetin in ethanolic extract from maceration is 118.43 ppm, while total quercetins from ethanolic extract with digestion technique at 60°C can rich up to 209.62 ppm. It suggests that the heat extraction method shown to have advantages of savings in time and energy to extract the quercetin compounds in *A. conyzoides* L. leaves successfully.

### Evaluation the suitable method that impart a high level of total yield extract and quercetin content

As described in Figure 1, the digestion techniques shown to have advantages of savings in time and energi, because this technique can produce a high total yield extract. Moreover, based on Figure 2, the digestion technique did not damage the compounds in extract. Furthermore, Figure 4 showed that the optimal total quercetin content (209.622 ppm) has obtained by digestion technique for two hours at 60 °C using ethanol as a solvent. Based on these results, it suggest the digestion technique for two hours at 60°C using ethanol as a solvent is the most efficient method to obtain the highest total yield extract and quercetin content in *A. conyzoides* L. leaves. Thus, this method will be used for further study to evaluate the chemical profile of *A. conyzoides* L. leaves.

### Application of TLC in analysis of phytochemical profiling of plant extract

A phytochemical profiling of an herbal medicine (HM) is a chromatographic pattern of the extract of some common chemical components of pharmacologically active and or chemical characteristics.<sup>22</sup> This chromatographic profile is an important step for the establishment of a consistent biological activity, a consistent chemical profile, or simply a quality assurance program for production and manufacturing of herbal drug.<sup>23</sup> Because of these parameters are crucial for preparation of accurate and potent, this work attempts to quantification of the biomarkers and/ or chemical markers and also to investigate chromatographic

phytochemicals profiles of *A. conyzoides* L. leaves extract. The phytochemicals profiling are shown in Figure 5.

In the current study, a phytochemicals profiling of *A. conyzoides* L. leaves extract were identified from three different origin of plant, there are Materia Medica from East Java, Balitro from West Java, and Purwokerto from Central Java. All of the areas of origin of plant are still in place in Indonesia country. The purpose of collecting plant in various areas is to collect the representative samples, so the phytochemicals profile which obtained from this experiment can demonstrate the profile of 70% ethanolic *A. conyzoides* L. leaves extract from all of area, especially in Indonesia. The extraction method in this study uses a digestion technique at 60°C for 2h and uses an ethanol as a solvent.

TLC profiles were shown in Figure 5 indicate that all sample constituent were clearly separated without any tailing and diffuseness and showed that all of sample extracts have the same profiles. Based on TLC profiles, there are many spots with different  $R_f$  values and the various of  $R_f$  values describe the presence of number of phytochemicals constituent in *A. conyzoides* L. leaves extract. The results showed that there are four the major spots can be seen clearly at 366 nm, among others M1, M2, M3, and M4. All of these spots can be used as a marker or as stability indicating methods for the determination of raw material or pharmaceutical formulations of *A. conyzoides* L. leaves extract.<sup>24</sup>

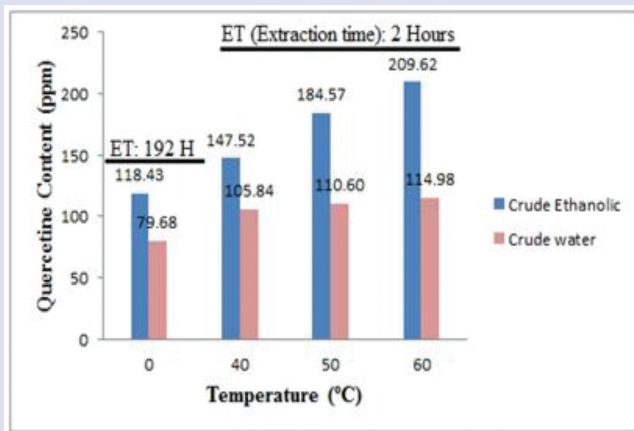
As previously explained, quercetin became as a marker compound of *A. conyzoides* L. leaves extract on this study because of many previous study has revealed the activity of quercetin from this plant. Marker compound means is a chemical constituent within a medicinal that can be used to verify its potency or identity. It is very difficult to identify correct marker compounds for all traditional medicinal, but using chromatographic fingerprint, the authentication and identification of herbal medicines can be accurately conducted.<sup>25</sup> Therefore, the use of markers and chromatographic fingerprinting techniques can give useful information assisting manufacturing control, minimising variations in production batches and assuring batch to batch consistency, with reproducible results.<sup>26</sup>

As shown in Figure 5, TLC profiles showed that the  $R_f$  value of all samples extract at 0.51 (M2) equal to the standard quercetin. It revealed the presence of quercetin in the sample extract. This result was confirmed by overlaying their UV absorbtion spectra with the reference standard as quercetin and scanned at 400 nm using a CAMAG TLC Scanner 3 integrated with winCATS software and the results shown in Figure 6.

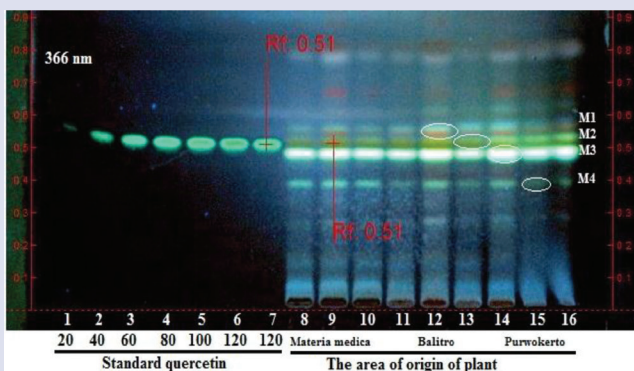
### Estimation of quercetin in 70% ethanolic leaves extract of *A. conyzoides* L. using TLC-densitometric method

Phytochemical profiles of plant extract are not adequate enough to ensure the quality of plant. Based on WHO guidelines in 2011 for the Quality Controls Methods for Herbal Medicine, there is a need quantitative analysis to ensuring the bioactive marker which has a responsible with therapeutic activity, so the activity of the extracts is consistent and reproducible in each product.<sup>27</sup> Therefore, this research also intended to evaluate the total quercetin in each of sample extract and the results were shown in Table 1.

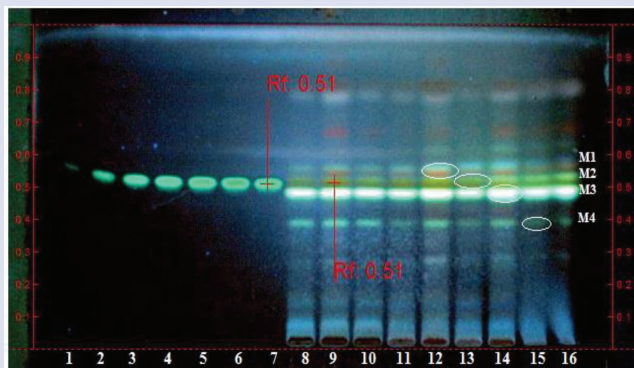
The results in Table 1 showed that the different location of plant can caused the different concentration of quercetin content. The ethanolic extracts from the leaves collected from Balitro exhibited the highest level of quercetin content (54.9165 ppm) followed by Materia Medica (52.7134) and Purwokerto (40.7134). However, the differentiation of the results is not too far. The quercetin content in each sample were measured by a graph was plotted taking concentration on X axis and absorbance on Y axis using standard quercetin (0 to 120 ppm). The linear correlation data of standard quercetin can see in Figure 7.



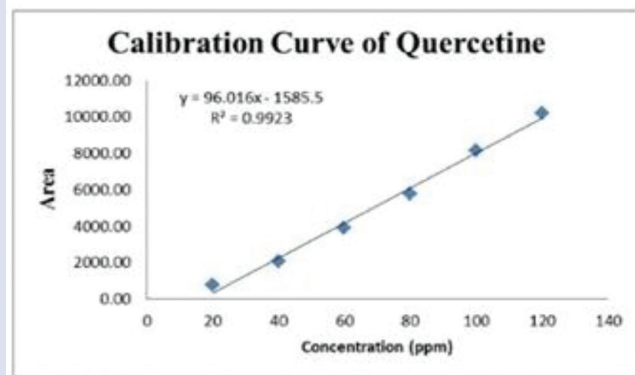
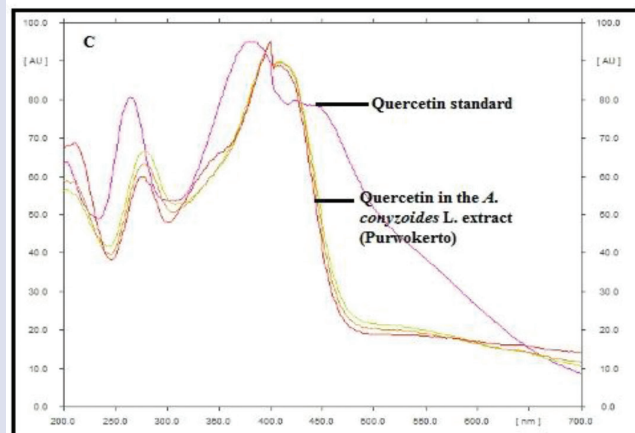
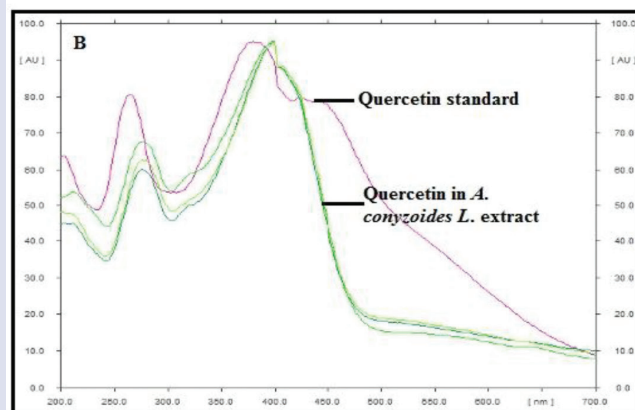
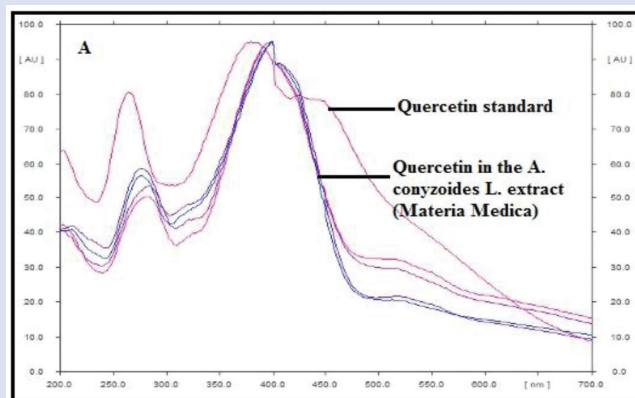
**Figure 4:** Estimation of the quercetin content from crude extract of *A. conyzoides* L. leaves



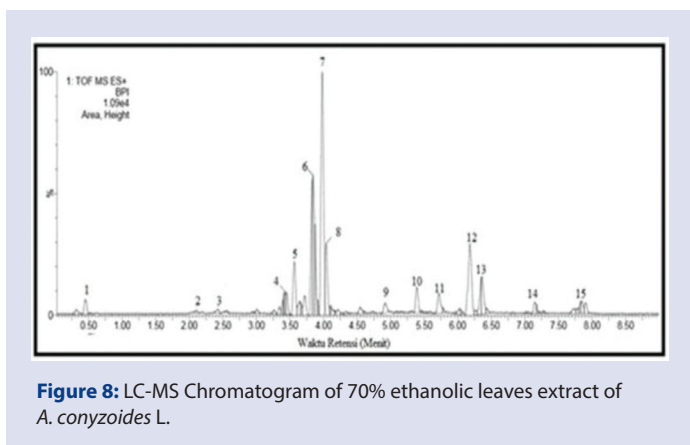
**Figure 5:** Phytochemical profiles of 70% ethanolic leaves extract of *A. conyzoides* L. leaves from various the area of origin of plant



**Figure 6:** TLC-densitometry chromatogram for 70% ethanolic leaves extract of *A. conyzoides* L. from several of origin of plant: A. The plant's source was from Materia medica (East Java); B. The plant's source came from Balitro (West Java); and C. The plant's source came from Purwokerto (Central Java).



**Figure 7:** The calibration curve of standard quercetin



**Figure 8:** LC-MS Chromatogram of 70% ethanolic leaves extract of *A. conyzoides* L.

**Table 1:** Estimation of quercetin content in 70% ethanolic leaves of *A. conyzoides* L

Source of Plant	Quercetin content (ppm)	%RSD
Purwokerto	40.7134	1.88
Balitra	54.9165	3.91
Materia Medica	52.7134	2.23

The quercetin content in Table 1 was determined from three experiments in on day (for intra-day precision) which are reported as %RSD. The intra-day precision as shown in Table 1 ranged from 1.88 to 3.91. According to the AOAC, the results demonstrate acceptable repeatability because the %RSD value did not exceed 5%.<sup>28</sup> Moreover, the linear regression equation as shown in Figure 8 was found to be  $Y = 96.016x - 1585.5$  with correlation coefficient ( $r$ ) 0.9923. The correlation coefficient was used as an indicator of linearity. Because of the correlation coefficient of this result high than 0.99, it indicates that the calibration

curve of standard quercetin has a high linearity relationship between area and concentration of standard quercetin.<sup>29</sup>

#### Application of LC-MS in analysis of phytochemical profiling of plant extract

Liquid chromatography separation the component of 70% ethanolic leaves extract of *A. conyzoides* L. was examined on Aquity UPLC BEH C18 column and the LC-MS chromatogram was shown in Figure 8. Tentative identification of phytochemical in samples extract was shown in Table 2.

As shown in Figure 8, a total of 11 compounds were identified by comparing molecular weight ( $M$ ) and  $m/z$  fragment with the literature data and the results were shown in Table 2. The results of the LC-MS analysis provide 15 major peaks determining the presence of phytochemical compounds, but only 11 compounds were identified. The results in Table 2 showed that ethanolic leaves extract of *A. conyzoides* L. has higher flavonoid content and the one of peak was identified as quercetin. As shown in Figure 8, quercetin was not a major component in 70% ethanolic extract of *A. conyzoides* L. leaves. The major component in the sample extract is kaempferol-7-O-rhamnopyranoside. This compound can be used as a chemical marker same as with quercetin.

#### Determination of the yield extract of 70% ethanolic leaves extract of *A. conyzoides* L.

Based on previous result, digestion method at 60°C for 2h using ethanol 70% as solvent is an effective method for the extraction of *A. conyzoides* L. leaves. Therefore, this method was used in this current study and the method were replicated in extraction process of plant from various location to obtain the standar of yield extract of 70% ethanolic leaves extract of *A. conyzoides* L. The results were described in Table 3.

As can be seen from the results in Table 3, a highest of yield extract was obtained from Materia Medica (16.60% w/w), followed by Balitra (15.75% w/w) and Purwokerto (14.15% w/w). Based on these results, the range of standard of yield extract is 14.15 to 16.60 %w/w. This result can be used as one of the parameters standards to development standardizes extract.

**Table 2:** Phytochemical constituent in 70% ethanolic leaves extract of *A. conyzoides* L. by LC-MS

Peak Number	$T_R$ (minutes)	$M$	$m/z$ Fragment	Tentatif Identification	Literature
1	0.454	301	620, 302	Quercetin-3-O-glycoside	(30)
2	2.111	343	366, 344	Hyperoside	(31)
3	2.422	348	412, 349	Unidentified	-
4	3.456	388	421, 389	Monohydroxy-penta-methoxy-flavone	(32)
5	3.563	418	441, 419	Kaempferol-3-O-pentose	(33)
6	3.828	402	425, 403	Hexa-methoxy flavone	(32)
7	3.980	432	455, 433	Kaempferol-7-O-rhamnopyranoside	(31)
8	4.046	416	434, 417	Eupalestin	(34)
9	4.966	216	313, 218	Luteoline	(35)
10	5.388	720	721, 369	Unidentified	-
11	5.767	608	609, 324	Quercetin-3-O-rutinoside	(36)
12	6.168	337	414, 338	Luteolin-7-O-glucuronide	(31)
13	6.383	440	482, 441	Apigenin	(31)
14	7.152	663	708, 664	Unidentified	-
15	7.868	509	554, 510, 170	Unidentified	-



**Table 3: The yield extract of 70% ethanolic leaves extract of *A. conyzoides* L. with various origin of plant**

Origin of Plant	Yield extract (%w/w)
Materia Medica (East Java)	16.60 ± 0.1000
Balitro (West Java)	15.75 ± 0.2500
Purwokerto (Central Java)	14.15 ± 0.0500

#### The methods for standardizing different batches for long Term from *A. conyzoides* L. leaves

As the explanation before, the natural product was influenced by many factors. Thus, the biggest challenge in the production of herbal medicines is obtained the quality, efficacy, and safety from herbal product and it should be reproducible. Therefore, there is need a tool to standardize the natural product from different bathes. According t the WHO guideline, there are many parameters should be checked to Development the standardize extract such as physicochemical parametes (yield extract, loss on drying, total ash value, etc), quantitative of bio-active marker, chromatographic pattern, etc. Based on the previous study, the range of standard of yield extract is 14.15 to 16.60 %w/w, while the range of quercetin content is 40.71 ppm to 52.71 ppm. Based on TLC profile, there are four major spot (M1, M2, M3, and M4), while based on LC-MS profile, quercetin and kaempferol can be used as marker compounds. All of these results can be used to identify proper quality of *A. conyzoides* L. leaves or can be used as a guideline for development of the standardize extract.

## CONCLUSIONS

The parameters of extraction process have an influence in the performance of the extraction process. However, our results suggest that the digestion technique for two hours at 60°C using ethanol as a solvent is the most efficient method to obtain the highest total yield extract and quercetin content in *A. conyzoides* L. leaves. The range of standard of yield extract and quercetin content in sample extract which combined by TLC and LC-MS profiles can be used to determine the proper quality of *A. conyzoides* L. leaves in different batches of herbs for long term.

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## CONFLICT OF INTEREST

We declare that we have no conflict of interest.

## ABBREVIATION USED

*A. conyzoides* L.: *Ageratum conyzoides* Linn.; **TLC**: Thin Layer Chromatography; **LC-MS**: Liquid Chromatography-Mass Spectroscopy; **DLBS**: Dexa Laboratories of Biomolecular Sciences; **UV-Vis**: Ultraviolet-Visible; **HM**: Herbal Medicine; **M1**: Marker 1; **M2**: Marker 2; **M3**: Marker 3; **M4**: Marker 4; **RSD**: (Relative Standard Deviation); **UPLC**: Ultra Performace Liquid Chromatography; **qTOF-MS**: Quadropole Time-of-Flight Mass Spectrometer; **ESI**: Electrospray Ionization; **v/v**: volume/volume; **µm**: mikro meter.

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## SUMMARY

- Digestion technique at 60°C can produce an extract with high yield extract and high of total quercetin.
- The solvent composition had an influence on TLC profile of *A. conyzoides* L. leaves extract, but the time and temperature of extraction have not.
- Based on TLC-densitometric quantification, total quercetin in crude ethanol higher than in crude water.
- Based on LC-MS profile, Kaempferol-7-O-rhamnopyranoside is the major component in the extract. In addition to Kaempferol-7-O-rhamnopyranoside, quercetin was also identified on LC-MS profile.
- The range of quercetin content in the leaves extract of *A. conyzoides* L. which combined with TLC and LC-MS profile can be used to determine the proper quality of plant material of *A. conyzoides* L. leaves.

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