The Jamu or herbal medicine were adulterated with chemical drugs in the last twenty years. One of the chemical drugs that are commonly added to herbal medicine is acetaminophen. Acetaminophen is generally considered an analgesic-antipyretic drug. The research aims to identify the content of paracetamol and its amount added to the jamu. This study consisted of 6 jamu or herbal medicine samples with different brands. The TLC method conducted the qualitative analysis using the mobile phase n-hexane: ethyl acetate (1:1). Three positive samples containing acetaminophen were obtained: D, E, and F. The three positive samples containing acetaminophen were calculated using the LCMS method. Quantitative analysis was conducted with LCMS using a reversed-phase with a mobile phase of acetonitrile: water (15%>: 85%> v/v) at a flow rate of 0.2 mL/min and an injection volume of 5 µL, it was obtained the levels of acetaminophen in sample D as much as 0.16 g/2 g of sample, in sample E of 0.63 g/7 g of sample, and sample F of 0.56 g/7 g of sample. Although the acetaminophen content in herbal medicine is relatively small, according to the Regulation of the Minister of Health of Indonesia No. 007 of 2012, traditional medicine or herbal medicine should not contain synthetic chemicals.

Keywords: Liquid Chromatography-Mass Spectroscopy (LCMS); Thin Layer Chromatography (TLC); Acetaminophen; Jamu; Herbal Medicines

Abstrak: Dalam dua puluh tahun terakhir, banyak jamu yang dipalsukan dengan obat-obatan kimia. Salah satu obat kimia yang biasa ditambahkan pada jamu adalah asetaminofen. Asetaminophen umumnya dianggap sebagai obat analgesik-antipiretik. Penelitian ini bertujuan untuk mengetahui kandungan parasetamol dan jumlah yang ditambahkan pada jamu. Sampel penelitian ini terdiri dari 6 sampel jamu dengan merek yang berbeda. Analisis kualitatif dilakukan dengan metode KLT menggunakan fase gerak n-heksana: etil asetat (1:1). Diperoleh tiga sampel positif mengandung asetaminofen, yaitu sampel D, E, dan F. Tiga sampel positif mengandung asetaminofen dihitung menggunakan metode LCMS. Analisis kuantitatif dilakukan dengan LCMS menggunakan fase terbalik dengan fase gerak asetonitril: air (15%>:85%> v/v) pada
The complexity of compounds in jamu or herbal medicine causes complexity in identifying herbs added to the chemical drugs or BKO due to the mixing of plant compounds. One solution to overcome the problems of the identification process is to use the LCMS method (Liquid Chromatography-Mass Spectroscopy) (Jian, 2017; Yang et al., 2020). This method combines two methods in one analysis system. The process of sample derivatization and the
calculation of levels is conducted using a chromatography system, UPLC (Ultra Performance Liquid Chromatography) (Chen et al., 2019; Niessen, 2017). Mass Spectroscopy was used to detect the compound by looking at the compound's fragmentation pattern and molecular weight (Trivedi et al., 2017). Compared with several other tandem mass spectrometries, it has advantages in detection sensitivity, mass accuracy, and resolution. In addition to that, the LCMS method is considered a fast, cost-effective, and straightforward method.

**Research Methods**

**Tool**

UPLC-MS (G2 QToF Waters), analytical balance (Shimadzu), 1 mL syringe, 10-100 µL micropipette, LC vials (waters), 3D shaker, glassware used in the UNG Pharmaceutical Chemistry laboratory.

**Material**

A total of 6 samples of Jamu in powder form were sold in the city of Gorontalo (A, B, C, D, E, F), acetaminophen standard, methanol pro analysis (MERCK), methanol hyper grade for HPLC (MERCK), ultrapure water (MERCK), acetonitrile hyper grade for LCMS (MERCK), n-hexane, ethyl acetate, 70% alcohol, Whatman filter paper.

**Procedures**

**Sample preparation (extraction)**

Each jamu or herbal medicine sample was weighed approximately 1 gram put into a beaker. Samples were extracted by maceration using 25 mL of methanol and then stirred at a constant speed of 150 rpm using a 3D shaker. The herbal medicine sample's liquid extract was filtered using Whatman filter paper, then collected in a beaker and evaporated until a viscous ache extract was obtained.

**TLC Testing**

The methanol extract of herbal medicine A and the compound of acetaminophen as a comparison were bottled on TLC plates with a size of 10 x 12 cm, put into a chamber containing eluent n-hexane: ethyl acetate (1:1). After the eluent reached the mark limit, it was removed and dried. Then the resulting chromatogram was stained under UV light 254 nm. The stains from the comparison compound were compared to each herbal extract. Then it was done with identification to reveal any similarity in the appearance of the stain, then the Rf value was calculated.

**Making a standard solution**

Acetaminophen has weighed as much as 10 mg, transferred into a 10 mL volumetric flask, and then dissolved with methanol and diluted to the acetaminophen content to 1000 ppm. And then, the sample was pipetted for 25 µL,
Preparation of sample solution

From the sample with a concentration of 1000 μg/mL, a solution of 5, 10, 15, 20, 25 μg/mL was made. This sample solution was pipetted as many as 25; 50; 75; 100; and 125 μL, each of which was put into a 5 mL volumetric flask and dissolved in methanol with the volume up to the marked line. Each standard solution was filtered and put into an LC vial. Then each concentration was injected into the system, and then a calibration curve and its regression equation were made.

Determination of level and preparation of LCMS tools.

Before the sample was separated, a program was set up to run the LC-MS tool. Samples were separated using a C\textsubscript{18} column with a temperature set at 25\textdegree{}C. The injection volume was 5 μL. Then the sample was brought to the mass spectrometer (ESI) for further qualitative and quantitative determination with a flow rate of 0.2 mL/min. The optimization data for LCMS conditions are as follows

Table 1. LCMS Conditions Optimization Data

| LCMS conditions optimization results |
|--------------------------------------|
| Types of elution systems              | Isocratic |
| Mobile phase                         | Acetonitrile: Water (15:85 v/v) |
| Column type                          | C\textsubscript{18} (Reversed-Phase Column) |
| Flow rate                            | 0.2 mL/minute |
| Injection volume                     | 5 μL |
| Mode of operation                    | Positive Ion (M +) |

Results and Discussion

Thin Layer Chromatography (TLC) Identification Results

Thin-layer chromatography is the simplest qualitative analysis method. According to Nazer et al. (2014), thin layer chromatography has significant advantages compared to paper chromatography. The stationary phase in TLC is a thin layer of an inert substance supported on a flat, unreactive surface. In addition to that, the sharpness of separation and detection sensitivity is even higher in TLC than in PC. The mobile phase used in the analysis is two solvent comparisons with different polarity levels, n-hexane: ethyl acetate at a ratio of 1:1. Optimization of the mobile phase in TLC is carried out to obtain the appropriate solvent based on a trial and error system (Coskun, 2016; Gandjar & Rohman, 2012; Wang et al., 2012).

Furthermore, the process of elution was carried out. The results were observed under UV 254 nm. Acetaminophen standard spots and aching rheumatic
pain Jamu samples can be seen at UV 254 nm due to the interaction between UV rays and the indicator on the plate, which is silica gel F254 (Komsta, 2016). The plate will glow in UV light 254, while the spots area will cover the light emitted by the plate so that the spots can be found (Fery et al., 2017; Sherma & Rabel, 2018). The results of the qualitative analysis by using the TLC method are as follows:

![TLC Chromatogram of Acetaminophen Standards and Jamu with a wavelength of 256 nm. Mobile phase n-hexane: ethyl acetate (1: 1)](image)

**Table 2.** Results of TLC Identification of Acetaminophen Standards and Jamu with a wavelength of 256 nm. Mobile phase n-hexane: ethyl acetate (1: 1)

| Aching Rheumatic Pain Jamu Sample | Rf Value (UV 254 nm) | Conclusion | Information |
|----------------------------------|----------------------|------------|-------------|
|                                  | Sample | PCT       |             |             |
| Jamu A                           | -      | -         | -           | It does not contain acetaminophen |
| Jamu B                           | -      | -         | -           | It does not contain acetaminophen |
| Jamu C                           | -      | 0.37      | -           | It does not contain acetaminophen |
| Jamu D                           | 0.37   | +         | Contains acetaminophen |
| Jamu E                           | 0.38   | +         | Contains acetaminophen |
| Jamu F                           | 0.38   | +         | Contains acetaminophen |

Based on the results of the analysis that has been carried out on 6 types of herbal samples, there are 3 types of herbs that have been identified as acetaminophen. The interpretation of the data for this qualitative analysis was made by calculating the retardation factor (Rf) on the intensity of the comparison standard spots and the six samples that appear. Rf is the ratio of the solute's distance and the eluting solution's distance on paper or thin film. The calculation
results obtained that the Rf value of acetaminophen standard as a comparison amounted to 0.37. The Rf value is still categorized as good Rf because, according to (Gandjar & Rohman, 2012), it is still included in the range of good Rf values, which is 0.2-0.8. Samples D, E, and F show the same Rf value as the acetaminophen standard as the comparison, namely 0.37, 0.38, and 0.38, respectively. The suitability of the Rf values of the comparison standard with the three samples of aching rheumatic pain Jamu indicated the presence of chemical drugs or BKO of acetaminophen in the aching rheumatic pain Jamu on the TLC method. However, to reinforce the suspicion of the three allegedly positive samples containing acetaminophen, the tests were carried out on the three samples using LCMS.

**Results of identification using LCMS**

The LCMS condition used in this study results from optimization, which is the initial stage before the instrument is used for quantitative analysis. The purpose of doing optimization of the LCMS tool is to determine the most suitable conditions in analyzing acetaminophen in herbal samples. Based on the optimization results of LCMS conditions (table 1), the best optimal conditions for analyzing acetaminophen are using isocratic elution systems. According to (Furusawa, 2019), eluents/so/vent with a constant composition in an isocratic elution system are pumped into the column during the analysis. The type of column used in this study is the C\textsubscript{18} column with a silica gel component and a reversed-phase column. According to (Marquis et al., 2017), silica octadecyl (ODS or C\textsubscript{18}) is the most used stationary phase because it can separate compounds with low, medium, or high polarity.

This analysis obtained a chromatogram of acetaminophen standard and each aching rheumatic pain Jamu sample that showed relatively the same retention time. According to (Georgelis et al., 2018), a qualitative analysis of LCMS is done by comparing the retention time of a pure compound with the retention time of the compound referred to in the sample. The chromatogram of acetaminophen standard and the three samples are as follows:
Figure 2. Comparison of Chromatograms of Acetaminophen and sample D, E, F

Table 3. Comparison of concentrations, retention times, acetaminophen standard, and samples

| Acetaminophen | Sample D | Sample E | Sample F |
|---------------|----------|----------|----------|
| C (µg/mL)     | Rf       | C (µg/mL) | Rf       | C (µg/mL) | Rf |
| 5             | 1.10     | 10       | 1.42     | 10       | 1.00 | 10       | 1.01 |
| 10            | 1.13     | 15       | 1.32     | 15       | 0.93 | 15       | 0.95 |
| 15            | 1.03     | 20       | 1.23     | 20       | 0.88 | 20       | 0.88 |
| 20            | 0.98     | 25       | 1.17     | 25       | 0.85 | 25       | 0.90 |
| 25            | 0.95     | 30       | 1.10     | 30       | 0.81 | 30       | 0.85 |

Based on the chromatogram in the figure above and the data are shown in table 3 about the comparison of concentration and retention time, and peak area of the chromatogram of acetaminophen standard and all three samples, the results showed that the average value of acetaminophen retention time was 1.038 minutes. Retention time is a measure of the time taken for a solute to pass through a chromatography column. It is calculated as the time from injection to detection, and the detector maximally captures the signal. The results of the comparison of the concentration and retention time of the chromatogram that appeared for the three samples showed a retention time that was relatively the same as acetaminophen, with an average value of 1 minute. The shift in retention time during the analysis is caused by the pressure that occurs in the column during the elution process. The data above shows that the retention time of the acetaminophen standard and the analyzed samples show the similarity of the retention time value. It was expected that herbal medicine contains the chemical drugs of acetaminophen.

Although retention times have indicated the compounds expected to be
present in the sample, it is still necessary to identify specific compounds using a mass spectrometer to analyze these compounds' ion molecules and fragmentation patterns. This aimed to prove that the compounds on the chromatogram are the compounds from the separation results. The mass spectrometer detector will identify the compound eluted from the LCMS column by ionizing it first then measuring the mass ratio (m/z) and molecular fragments into small pieces. The results of detection of acetaminophen with a mass spectrometer are as follows:

![Mass Spectra of Acetaminophen and its fragment substituents.](image)

**Figure 3.** Mass Spectra of Acetaminophen and its fragment substituents.

**Table 4.** Comparison of Molecular Weight and m/z values of LCMS instruments

| Sample | Molecular Weight (g/mol) | Fragment Mass Ratio (m/z) |
|--------|--------------------------|---------------------------|
|        | Sample | PCT | Sample | PCT |
| Sample D | 152.73 | 152.36 | 81.96 | 152.73 | 81.81 | 152.36 |
| Sample E | 152.17 | | 83.35 | 152.17 |
| Sample F | 152.13 | | 80.32 | 152.13 |
The data above shows the detected molecular weight and fragmentation patterns. The molecular weights of acetaminophen detected were 152, 109, and 81 m/z (presented in figure 3 and table 4). Two fragments formed from acetaminophen, namely p-aminophenol (109 m/z) and acetaldehyde (44 m/z). The transfer of protons in the methyl group of acetaldehyde to nitrogen ions in the p-aminophenol group breaks the bonds. As a result of the aromatic resonance process in p-aminophenol ions, there was a gap of bond termination. It results in aldehyde fragments (32 m/z) and cyclopentadienyldene (81 m/z) (Geib et al., 2019; Lu et al., 2018). Molecular weight obtained underwent a shift in molecular weight values from 151.16 g/mol to 152.36 g/mol due to the mode of operation used in mass spectrometer detectors, positive ion mode (M+). According to (Zhang et al., 2020), M+ indicates that the molecular ion carries a positive charge because it has lost one electron, so the resulting molecular weight increases by one (1).

After obtaining mass spectra and chromatograms, both on the acetaminophen standard and all three samples, the next step is to analyze the acetaminophen levels in herbal samples. Chromatograms that show responses in peak areas can be used for quantitative analysis. The results of the examination of acetaminophen levels are as follows:
The results of quantitative analysis (table 5) showed that all three samples contained acetaminophen. This is evident from the overall response of the chromatogram area of each sample.

A validation test was performed to prove that the parameters used can meet the requirements. According to (Moreton, 2016), method validation is carried out to ensure that the analytical method is accurate, specific, reproducible, and resistant to the range of analytes to be analyzed. The parameters used in this test include accuracy, precision, the limit of detection and limit of quantitation, and linearity.

| Parameter                  | Results     | Terms      | Information |
|----------------------------|-------------|------------|-------------|
| Accuracy                   | 100.29%     | 98-102%    | Qualified   |
| Precision                  | 0.7048      | SD <2      | Qualified   |
| Linearity                  | $r^2 = 0.9816$ | $r^2 > 0.9000$ | Qualified |
| Limit of Detection (LOD)   | 0.16 µg/mL  | -          | -           |
| Limit of Quantification (LOQ) | 0.50 µg/mL | -          | -           |

Based on the table above, the result of the data obtained was 100.29%. This shows a good recovery value (% recovery) because the accuracy requirement, according to Harmita (2004), is 98% -102%. The result of the linearity of the five variations of concentration was made from the range of 10-30 ^g/mL, the value of the area obtained was plotted into the y-axis. In contrast, the standard series was plotted into the x-axis so that a calibration curve was created with the line equation of $y = 0.14x + 6.84$. From this equation, the value of a is an intercept whose result was amounted to 6.84, which means that the curve intersects the y-axis at point 6.84. While the value of b = 0.14, which represents the slope value of the curve. Value of $r^2 = 0.9816$. The value of r is the value of the correlation coefficient. According to (Borman & Elder, 2017; Gazioğlu & Kolak, 2015), the condition for accepting the correlation coefficient is the value of r > 0.900. Thus, the linearity parameter data shows a linear relationship between the levels and the peak area.

From the test results on the precision test, SD precision amounted to 0.7048. In the precision set, acceptance criteria are SD<2 (Borman & Elder, 2017; Gazioğlu & Kolak, 2015). This shows that the precision test results are still included in the criteria for acceptance of SD values. In addition to the three
validation parameters tested, the most recent determination of validation parameters is the limit of detection (LOD) and the limit of quantification (LOQ). From the calculation results, the detection limit amounted to 0.16 µg/mL, and the limit of quantification amounted to 0.50 µg/mL.

Conclusion

This LCMS method can determine complex compounds in herbs compared to acetaminophen. Based on the results of the analysis of acetaminophen from six samples analyzed using the LCMS method, the results obtained were three samples that were proven to contain the chemical drugs of acetaminophen. The level obtained for samples D, E, and F, respectively, amounted to 0.16 g/2 g sample, 0.63 g/7 g sample, and 0.56 g/7 g sample. The analysis results based on the LCMS method showed that there were still chemical drugs found in herbal medicine. Moreover, the LCMS method can be used in samples containing complex compounds.

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