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Optimization of Thin-Layer Chromatography Mobile Phase and Phytochemical Identification of the Ethyl Acetate Fraction of Houttuynia cordata (H. cordata) Leaves and Rhizomes

Optimasi Fase Gerak Pada Kromatografi Lapis Tipis dan Identifikasi Fitokimia Fraksi Etil Asetat Daun dan Rimpang *Houttuynia cordata* (*H. cordata*)

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ABSTRACT

Houttuynia cordata (H. cordata) is a medicinal plant that contains various secondary metabolites, especially flavonoids that have potential biological activity. This study aims to optimize the mobile phase and evaluate the chromatographic profile of ethyl acetate fractions of H. cordata leaves and rhizomes using TLC. Separation was performed on a silica gel stationary phase with several mobile phase variations, n-hexane: ethyl acetate (3:7) and dichloromethane : ethyl acetate (6:4). The results showed that the n-hexane and ethyl acetate (3:7) mobile phase provided the most optimal separation. The leaf fraction was identified to contain quercetin, quercitrin, and kaempferol, while the rhizome fraction was identified to contain quercetin and kaempferol. In conclusion, the TLC method with n-hexane and ethyl acetate (3:7) as the mobile phase is effective for separating and profiling semipolar compounds in the ethyl acetate fraction of H. cordata and has the potential to support further research.

Keywords: Houttuynia cordata, Thin layer chromatography, TLC mobile phase, Phytochemical.

ABSTRAK

Houttuynia cordata (H. cordata) merupakan tanaman obat yang mengandung berbagai metabolit sekunder, terutama flavonoid yang memiliki potensi aktivitas biologis. Penelitian ini bertujuan untuk mengoptimasi fase gerak serta mengevaluasi profil kromatografi fraksi etil asetat daun dan rimpang H. cordata menggunakan KLT. Pemisahan dilakukan pada fase diam silika gel dengan beberapa variasi fase gerak, yaitu n-heksana : etil asetat (3:7) dan diklorometana : etil asetat (6:4). Hasil penelitian menunjukkan bahwa fase gerak n-heksana : etil asetat (3:7) memberikan pemisahan yang paling optimal. Fraksi daun teridentifikasi mengandung kuersetin, kuersitrin, dan kaempferol, sedangkan fraksi rimpang teridentifikasi mengandung kuersetin dan kaempferol. Kesimpulannya, metode KLT dengan fase gerak n-heksana : etil asetat (3:7) efektif untuk memisahkan dan memprofilkan senyawa semipolar dalam fraksi etil asetat H. cordata serta berpotensi mendukung penelitian lebih lanjut.

Kata kunci: Houttuynia cordata, Kromatografi Lapis Tipis, fase gerak KLT, fitokimia

INTRODUCTION

The Saururaceae family includes the plant *Houttuynia cordata* (*H. cordata*). The mountains of China, Japan, Korea, and Southeast Asia are home to this herbaceous plant (Fu *et al*., 2013). *H. cordata* is known as fishwort or heartleaf, rhizomatous herb, due to its distinctive fishy smell and heart-shaped leaves. The plant typically grows to a height 30-60 cm and produces greenish yellow flowers with tree stamens. Rhizomes are slender and purplish-red, the seeds are dicotyledonous and sterile, the perioles are pubescent, the peduncles are sloghhtly pubescent, the nods are glabrous, and the apical stem grow upright (Asakawa *et al*., 2017; Laldinsangi, 2022). (**Figure 1.**)

Figure 1. *Houttuynia cordata Thunb* (1.); Leaves of *H. cordata* (2.) Rhizomes of *H. cordata*

H. cordata has been used as a medicinal plant in traditional medicine, relying on the plant and its extracts (Kumar *et al*., 2014; Pradhan *et al*., 2023; Song *et al*., 2021). *H. cordata* exhibits varying component content between the upper and lower layers of the soil surface. A study conducted by Verma's team found that the upper layer contained a higher concentration of components compared to the lower layer (Verma *et al*., 2017). The rhizome is used to treat pneumonia and lung abscesses, as it contains bioactive compounds such as alkaloids, flavonoids, volatile compounds, organic acids, polyphenols, and fatty acids. (Luo *et al*., 2022; Xiang *et al*., 2019). A study examining the nutritional and phytochemical profile of *H. cordata* found that it includes essential minerals and various active compounds, notably flavonoids and volatile oils. (Asakawa *et al*., 2017; Pradhan *et al*., 2023; Sharma *et al*., 2023).

Phytochemical studies can be conducted using TLC, which is a chromatography technique used in the qualitative analysis of chemical compounds because it is simple, rapid, and relatively economical (Kharnaior and Thomas, 2020; Modi and Shah, 2023). TLC is generally applied as a separation process, and separation in this method is greatly influenced by the interaction between the stationary phase and the mobile phase.

The selection of the mobile phase (eluent) is very important in the separation of compounds, because if it is not suitable, it can cause the compounds to not move from the starting line or move too quickly, resulting in suboptimal separation conditions. Therefore, optimization of the mobile phase in TLC is carried out by varying the type and ratio of solvents. Previous studies on the phytochemistry of active compounds using TLC usually started from crude extracts, but phytochemical studies on fractions are still limited. Therefore, this study conducted fractionation of the extract and TLC testing by optimizing the mobile phase.

METHODS

Tools and Materials

Tools

Laboratory glassware, dropper pipettes, analytical balance (Ohaus 214), SpeedVac SPD 20 (Thermo Scientific), Liquid Vacuum Chromatography Column, Rotary Evaporator (Heidolph L400), Water Bath (Mammert), Acid Cabinet (I-blush), Vacuum Pump, UV Lamp (Camag), and Chamber (Camag).

Materials

The materials used were *H. cordata* leaves and rhizomes; 70% ethanol, ethyl acetate p.a (Smart-Lab), silica gel 60 G for thin layer chromatography (Merck), silica gel 60 F254 (Merck), capillary tubes, and filter paper.

Extraction and Fractionation

The extraction process uses the maceration method with 70% ethanol solvent for 3 x 24 hours in a glass container until all the simplisia is completely submerged. The resulting macerate is concentrated using a rotary evaporator at a temperature of ± 50-60 ºC, then evaporated using a water bath to obtain a thick extract. The yield of the extract obtained is calculated using the following formula:

$$\%Yield = \frac{Final\ sample\ mass}{Initial\ sample\ mass} x 100\% \ (1)$$

The fractionation steps were carried out using liquid vacuum chromatography or column chromatography. The column was first prepared by inserting filter paper at the end of the column, then filled with 50 g of silica gel 60 F254 stationary phase, which was added gradually and compacted. The sample, consisting of ethanol extracts of *H. cordata* leaves and rhizomes, is impregnated with silica gel 60 F254 before being placed in the column. The elution process is carried out by pouring the mobile phase, ethyl acetate. The fractions are collected and then evaporated using a rotary evaporator until a thick fraction is obtained. Each fraction is then stored in a vial.

Identification of phytochemicals using TLC

Preparation of TLC and mobile phase

A silica gel plate measuring 10 x 1 cm was prepared and marked with a lower boundary line and an elution end line 1 cm from the bottom. The mobile phase used was n-hexane and ethyl acetate in a ratio of (3:7, 5:10). Each mobile phase is placed in the chamber and allowed to saturate for 30 minutes.

Testing with TLC

The ethyl acetate fractions of *H. cordata* leaves and rhizomes are spotted on the bottom line of the TLC plate with a capillary tube and then dried. The TLC plate is then placed in a chamber containing the mobile phase. The plate is removed from the chamber when the solvent reaches the upper limit. The TLC plate was allowed to dry, then observed under UV light at λ 254 and 366 nm for the appearance of colorless and non-fluorescent spots. The Rf value was calculated using the following formula:

$$Rf = \frac{Spot\ distance}{Mobile\ phase\ distance} \quad (2)$$

Results And Discussion

Extraction And Fractionation

The extraction yield of *H. cordata* leaves and rhizomes was 16.09% for leaves and 10.06% for roots. The selection of the maceration method was due to its suitability for use in cases of heat-sensitive or thermolabile plant chemical compounds (Julianto, 2019). The most effective solvent for extracting *H. cordata* was found to be 70% ethanol, which can extract a variety of chemicals, including phenolics, terpenoids, sterols, and alkaloids. This is consistent with earlier research that used water plus 96%, 70%, 50%, and 30% ethanol for extraction (Hung *et al*., 2023; Lee *et al*., 2015). This process involves the immersion of organic compounds found in the sample, which will diffuse through the cell wall.

The extract is then fractionated using vacuum liquid chromatography (VLC) or column chromatography. Fractionation of the *H. cordata* ethanol extract is carried out based on the principle of differences in polarity and molecular weight between solvents for the fractions to be used. The purpose of fractionation with ethyl acetate solvent is because this solvent is a semi-polar solvent that can attract secondary metabolites. The results obtained from the ethyl acetate fraction of the leaves and rhizomes were greenish-black in color, weighing 0.66 grams and 0.68 grams, respectively.

Optimization and Identification of Phytochemicals

The obtained results showed significant differences in the number of spots, retention factor (Rf) values, and the distance between separated spots. The optimization of the mobile phase in the TLC method was performed to achieve optimal compound separation based on differences in polarity and the affinity of compounds toward the stationary phase. In this study, two mobile phase systems were used: n-hexane : ethyl acetate (3:7) and dichloromethane : ethyl acetate (6:4). The variation in mobile phase composition was intended to adjust the polarity level of the solvent system so that differences in the affinity of individual compounds toward the stationary phase could be more clearly observed.

Based on the results obtained for mobile phase 1 (**Figure 2**), namely dichloromethane and ethyl acetate (6:4), observation under UV 254 nm showed that each fraction (leaf and rhizome) produced a distinct spot, with Rf values of 0.5 for the leaf fraction and 0.25 for the rhizome fraction. The Rf value of 0.5 in the leaf fraction was close to the reported Rf value of standard quercetin under UV 254 nm (Sainthanpari et al., 2023). The slight difference in Rf values may be attributed to variations in the mobile phase system used. Therefore, this spot was tentatively suggested to correspond to quercetin.

Furthermore, observation under UV 366 nm revealed six spots in the ethyl acetate leaf fraction with Rf values of 0.31, 0.37, 0.56, 0.72, 0.81, and 0.93. In contrast, the rhizome fraction showed four spots with Rf values of 0.31, 0.47, 0.56, and 0.72. The spot with an Rf value of 0.93 in the leaf fraction was comparable to the reported Rf value of standard quercetin (0.98) as described by Sriya et al., (2011). The small discrepancy in Rf values is likely influenced by differences in the mobile phase composition used in the analysis.

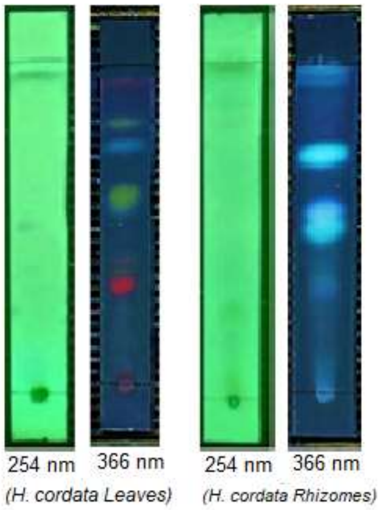

Figure 3. Using mobile phase diclorometana : ethyl acetate (6:4)

The optimization of the second mobile phase system (**Figure 3**), n-hexane : ethyl acetate (3:7), resulted in improved chromatographic separation. Under UV 254 nm, the leaf fraction exhibited two distinct spots with Rf values of 0.06 and 0.85, while the rhizome fraction showed three spots with Rf values of 0.56, 0.68, and 0.85. Observation under UV 366 nm revealed a higher number of detectable spots. The leaf fraction presented eight spots with Rf values of 0.28, 0.33, 0.43, 0.53, 0.58, 0.68, and 0.78, whereas the rhizome fraction showed six spots with Rf values of 0.56, 0.67, 0.71, 0.78, 0.85, and 0.912. A spot in the leaf fraction with an Rf value of 0.43 (observed under UV 366 nm) was consistent with the reported Rf value of quercitrin described by Hung et al., (2023). Moreover, the spots with Rf values of 0.68 in the leaf fraction and 0.67 in the rhizome fraction correspond closely to the reported Rf value of standard kaempferol (0.67) as noted by Pakyntein, (2023).

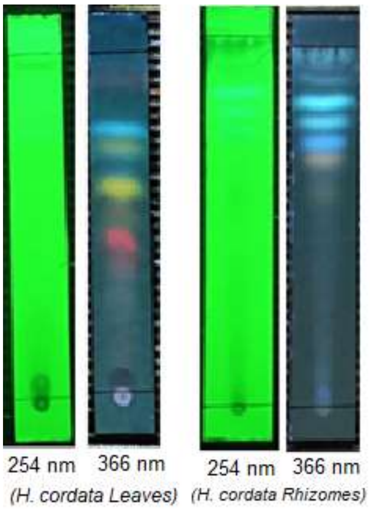

Figure 2. Using mobile phase n-hexane : ethyl acetate (3:7)

Quercetin and quercitrin can be detected under UV light at 254 nm and 366 nm due to the presence of chromophores and auxochromes within their flavonoid structures. Both compounds possess a flavonol backbone (C6–C3–C6) with an extensive conjugated double bond system ($\pi \rightarrow \pi^*$), particularly in the aromatic rings and carbonyl group, which functions as the primary chromophore responsible for UV absorption (Materska, 2008).

Under UV 254 nm, TLC plates containing a fluorescent indicator emit a green background, while quercetin and quercitrin appear as dark spots. This occurs because these compounds absorb UV radiation at 254 nm via $\pi \rightarrow \pi^*$ electronic transitions, thereby quenching the fluorescence of the plate.

In contrast, under UV 366 nm, both compounds exhibit visible fluorescence. This fluorescence is enhanced by the presence of auxochromic groups such as hydroxyl (–OH) groups attached to the aromatic system, which facilitate electronic transitions ($n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$) and increase emission intensity. Quercetin generally shows stronger fluorescence compared to quercitrin due to its aglycone form, which contains more free hydroxyl groups. In quercitrin, the hydroxyl group at the C-3 position is substituted with a rhamnose sugar, slightly reducing its fluorescence intensity.

Besides quercetin and quercitrin, the chromatographic pattern suggests the potential occurrence of other flavonols such as kaempferol. Kaempferol (3,4′,5,7-tetrahydroxyflavone) shares the typical flavonol skeleton but differs from quercetin in its hydroxyl substitution pattern, containing one fewer hydroxyl group on the B-ring. This structural variation slightly decreases its polarity and may affect both its Rf value and UV detection behavior. Kaempferol is widely recognized for its antioxidant, anti-inflammatory, cardioprotective, and anticancer properties, and its possible presence further supports the abundance of flavonol compounds in the ethyl acetate fractions.

Under UV 254 nm observation, the TLC plate displays dark black spots against a bright green background. This phenomenon occurs due to the interaction between UV radiation and chromophore groups associated with auxochromes in the compounds. In contrast, under UV 366 nm, chromophore groups produce more distinct fluorescent spots that are clearly distributed across the plate. This indicates that chromophores are functional groups responsible for color formation (Husna and Mita, 2020).

In the second mobile phase system, n-hexane : ethyl acetate (3:7), the eluent composition contains a higher proportion of ethyl acetate, which is polar, compared to the non-polar n-hexane. Consequently, the overall polarity of this mobile phase is relatively higher than that of the first system. This condition is appropriate because the ethyl acetate fraction contains semi-polar secondary metabolites such as flavonoids and phenolic compounds. These compounds possess hydroxyl (-OH) groups capable of forming hydrogen bonds with silica gel, the polar stationary phase.

Overall, the identification of compounds in this study remains tentative, as it is based on similarities in Rf values and visual characteristics under UV light. Definitive structural confirmation requires further analysis using advanced techniques such as LC-MS, FTIR, or NMR. Nevertheless, the optimization results indicate that the n-hexane : ethyl acetate (3:7) system is the most effective mobile phase for profiling or preliminary screening of flavonoid compounds in the ethyl acetate fractions of leaves and rhizome.

CONCLUSION

Based on the results of the study, it can be concluded that the optimization of the n-hexane and ethyl acetate (3:7) motion phase can be considered the most optimal eluent because it provides good separation of flavonoid compounds in the ethyl acetate fraction of *H. cordata* leaves and rhizomes. Phytochemical identification showed the presence of quercetin, quercitrin, and kaempferol compound.

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