Khairunnisa et al (2026)
Vol 14(1): 18634
https://doi.org/10.20884/1.api.2026.14.1.18634

Acta Pharmaciae Indonesia:
Acta Pharm Indo
E-ISSN 2621-4520

RESEARCH ARTICLE Open Access

Four of five *Kleinhovia hospita* leaf extracts meet moisture and microbial limits, with a quercetin-consistent TLC marker only in the ethyl acetate extract

Khairunnisa*, Indah Solihah, Herlina
Department of Pharmacy, Faculty of Mathematics and Natural Sciences, Universitas Sriwijaya, Indralaya, Ogan Ilir 30662, South Sumatra, Indonesia
*Corresponding author: Khairunnisa, Department of Pharmacy, Faculty of Mathematics and Natural Sciences, Universitas Sriwijaya, Jl. Raya Palembang–Prabumulih Km 32, Indralaya, Ogan Ilir 30662, South Sumatra, Indonesia. E-mail: khairunnisanisa249@gmail.com

ABSTRACT

Background: *Kleinhovia hospita* Linn. (tahongai) leaves are used in Indonesian traditional medicine, but quality specifications for their extracts are not yet defined. Extracts prepared with different solvents have not been compared using the same quality parameters.

Objective: To compare the specific and non-specific quality parameters of five tahongai leaf extracts prepared with solvents of different polarity.

Methods: Five extracts of leaves from Belitang, South Sumatra, were compared: three sequential macerates (n-hexane, ethyl acetate, residual ethanol) from our earlier cytotoxicity study, a single 96% ethanol macerate, and an infusion. The extracts were evaluated for thin layer chromatography (TLC) detection of quercetin and kaempferol, extractive values, specific gravity, loss on drying, moisture, ash values, and microbial counts. Moisture (≤10%) and microbial limits followed Indonesian regulatory requirements.

Results: Single maceration and infusion gave yields of 32.36% and 44.61%, respectively. The 96% ethanol extract had the highest ethanol-soluble extractive (35.87%) and contained alkaloids, flavonoids, tannins, saponins, and steroids. Four of five extracts met the moisture limit (3.37–9.42%), whereas the infusion did not (17.33%). All extracts met the microbial limits (total plate count ≤1.2×10^3 CFU/g; mould and yeast ≤5.8×10^2 CFU/g). Only the ethyl acetate extract showed a spot consistent with quercetin (R_f 0.32 vs 0.35), and kaempferol was not confirmed in any extract.

Conclusion: Solvent polarity determined the composition and quality of tahongai leaf extracts. The 96% ethanol and ethyl acetate extracts are the most suitable candidates for further standardization, which will require replicate data and quantitative marker assays.

Keywords: extract standardization, *Kleinhovia hospita*, quality control, quercetin, solvent polarity, thin-layer chromatography

Introduction

Kleinhovia hospita L. (Malvaceae), known in Indonesia as *tahongai*, is a small tree distributed across Southeast Asia and the Pacific. Its leaves are used traditionally to treat liver disease, and leaf extracts show strong antioxidant activity [1]. In South Sumatra, the Komering community uses the leaves for inflammation, tumours, and skin complaints [2,3].

Phytochemical studies have isolated cycloartane triterpenoids and unusual triterpenoid alkaloids, the

Received: December 13, 2025; Revised: October 20, 2026
Accepted: October 20, 2026; Published: October 21, 2026

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kleinhospitines, from this plant [4,5]. Some of these compounds showed hepatoprotective, antiproliferative, or anti-HIV activity *in vitro* [4,5]. Leaf extracts have also reduced doxorubicin-induced liver and heart toxicity in rats [6], and have shown antibacterial [7,8] and antiplasmodial activity [9].

These activities make tahongai a candidate raw material for herbal products, but such use requires defined quality specifications. Our group has standardized one ethanolic extract of tahongai leaves [2] and screened sequential extracts for cytotoxicity [3]. However, no study has compared extracts made with solvents of different polarity using the same set of quality parameters. The Indonesian Herbal Pharmacopoeia also has no monograph for this plant [10]. Without such data, it is unclear which extract type is the most suitable starting point for a standardized product.

This study therefore compared five tahongai leaf extracts prepared by sequential maceration, single maceration, and infusion. We evaluated their specific and non-specific quality parameters and their compliance with Indonesian moisture and microbial limits.

Methods

Research design

This laboratory experimental study compared five extracts of *K. hospita* leaves. Quality parameters were determined with the general methods of the Indonesian Herbal Pharmacopoeia [10]. Ethical approval was not required because the study involved no human participants or animals.

Plant material

The leaves came from the same source described in our previous reports [2,3]. Briefly, they were collected in Belitang, Ogan Komering Ulu District, South Sumatra, Indonesia, and identified at the Herbarium of Andalas University (ANDA) (letter No. 332/K-ID/ANDA/VIII/2017). The leaves were washed, sorted, air-dried in the shade, and ground into powder.

Extraction

Single maceration with 96% ethanol. Dried leaf powder (500 g) was macerated in 6 L of 96% ethanol (Dira Sonita®) for 72 h. The marc was re-macerated three times with fresh 6 L portions of solvent. The combined filtrates were concentrated in a rotary evaporator (Yamato®) at 65 °C to obtain a thick extract.

Sequential maceration. Dried leaf powder (1,000 g) was macerated in 6 L of n-hexane (Brataco®). The solvent was replaced every 2 days until the macerate was colourless, which took about 10 days. The marc was then macerated in the same way with 6 L of ethyl acetate (Brataco®) and finally with 6 L of 96% ethanol. Each filtrate was concentrated in a rotary evaporator [3]. The ethanol extract from this sequence is called the residual ethanol extract, to distinguish it from the single-maceration extract. These three extracts are the same extracts whose yields, phytochemical profiles, and cytotoxicity were reported previously [3]; those data are not repeated here.

Infusion. Dried leaf powder (200 g) was boiled with 2 L of distilled water (Brataco®; 1:10, w/v) for 15 min [10]. The infusion was filtered and freeze-dried (Labconco®).

Yield was calculated as the mass of extract divided by the mass of dried leaf powder used, multiplied by 100%.

Phytochemical screening

The 96% ethanol extract and the infusion were screened with the tube tests described previously [2,3,11,12]. Flavonoids were detected with magnesium powder (0.2 g) and concentrated hydrochloric acid (red to orange-red colour). Saponins were detected as a stable foam after a boiled aqueous mixture was shaken vigorously. Alkaloids were extracted with ammonia–chloroform and 2 N sulfuric acid and detected with Mayer (white precipitate), Wagner (brown precipitate), and Dragendorff reagents (orange-red precipitate). Steroids and triterpenoids were detected with the Liebermann–Burchard reagent (green or blue for steroids; red or purple for triterpenoids), and tannins with 1% iron(III) chloride (dark blue or greenish-black colour). All screening reagents were obtained from Merck®.

TLC identification of quercetin and kaempferol

The extracts and the quercetin and kaempferol reference standards (Andalas University) were prepared at 5% (w/v) and spotted side by side on silica gel 60 F_{254} plates (Merck®; 7 × 10 cm, 1 cm margins). Plates were developed with chloroform–ethyl acetate (5:2, v/v), as in our previous report [3]. Spots were examined under UV light at 254 and 366 nm and after spraying with 5% aluminium chloride in ethanol [13]. The retardation factor (R_f) was calculated as the distance travelled by the spot divided by the distance travelled by the

solvent front. A compound was considered tentatively present when an extract spot matched the standard in R_f (±0.05) and in colour after aluminium chloride spraying. R_f values were measured on the archived plate photographs.

Specific parameters

Ethanol-soluble extractive. One gram of extract, weighed on an analytical balance (Ohaus®; readability 0.0001 g), was shaken with 25 mL of 96% ethanol in a stoppered flask. The flask was shaken frequently for 6 h and then left to stand for 18 h. The mixture was filtered rapidly to prevent evaporation, and 5 mL of filtrate was evaporated to dryness in a tared dish. The residue was dried at 105 °C to constant weight. The extractive value (%) was calculated as residue mass × 5 divided by sample mass × 100.

Water-soluble extractive. The procedure was the same, except that chloroform water (2.5 mL of chloroform in 1 L of water) replaced ethanol [10].

Specific gravity. A calibrated pycnometer (Pyrex®) was weighed empty and filled with freshly boiled water at 25 °C. The extract was diluted to 5% (w/v), brought to 25 °C, and weighed in the same pycnometer. Specific gravity was calculated as the mass of the diluted extract divided by the mass of water of equal volume.

Non-specific parameters

Loss on drying. One gram of extract was weighed into a covered crucible previously dried at 105 °C for 30 min. The extract was dried uncovered at 105 °C to constant weight. Loss on drying (%) was calculated as mass lost divided by initial mass × 100 [10].

Moisture content. Moisture content was measured with a halogen moisture analyzer (Adam®).

Total ash. One gram of extract was placed in a tared crucible and ignited in a furnace (Daihan®) with a gradual temperature increase to 600 ± 25 °C until free of carbon. Total ash (%) was calculated relative to the sample mass.

Acid-insoluble ash. The total ash was boiled with 25 mL of dilute sulfuric acid for 5 min. The insoluble matter was collected on ashless filter paper, washed with hot water, ignited at 600 ± 25 °C, and weighed. Acid-insoluble ash (%) was calculated relative to the sample mass.

Total plate count. One gram of extract was diluted to 10 mL with 0.1% peptone water (peptone, Sigma-Aldrich®) (10^{-1}) and serially diluted to 10^{-6}. One millilitre of each dilution was pipetted into duplicate sterile Petri dishes, and 15 mL of nutrient agar (Sigma-Aldrich®) was poured and mixed. Media and diluents were sterilized in an autoclave (Yazumi®), and plating was performed in a laminar air flow cabinet (Biobase®) with micropipettes (DragonLab®). Plates were incubated inverted in an incubator (Biobase®) at 35–37 °C for 24–48 h. Counts were expressed as colony-forming units per gram (CFU/g) [14,15].

Mould and yeast count. One gram of extract was diluted to 10 mL with sterile diluent of 0.05% agar (Sigma-Aldrich®) in distilled water (10^{-1}) and serially diluted to 10^{-4}. A 0.5 mL portion of each dilution was spread on potato dextrose agar (PDA; Sigma-Aldrich®) plates in duplicate. Plates were incubated at 20–25 °C for 5–7 days, and colonies were counted and expressed as CFU/g. Media and diluent blanks were included as sterility controls.

Acceptance criteria and data analysis

Because no pharmacopoeial monograph exists for *K. hospita* [10], compliance was judged only for parameters with general Indonesian regulatory limits. Moisture content was compared with the 10% limit for powdered and solid oral traditional medicines in BPOM Regulation No. 32 of 2019 [14]. The same regulation was used for the microbial limits for oral traditional medicines, namely a total plate count $\leqslant 10^5$ CFU/g and a mould and yeast count $\leqslant 10^3$ CFU/g [14]. Extractive values, specific gravity, loss on drying, and ash values have no applicable limit and are reported descriptively as candidate specifications. Each extract was analysed as a single preparation, and microbial counts are means of duplicate plates. Because replicate-level data were not available for the other parameters, results are presented descriptively without inferential statistics.

Results

Extraction yield

Single maceration of 500 g of leaf powder with 96% ethanol gave 161.82 g of thick extract (yield 32.36%). Infusion of 200 g of leaf powder gave 89.22 g of freeze-dried extract (yield 44.61%).

Phytochemical screening

The 96% ethanol extract contained alkaloids, flavonoids, tannins, saponins, and steroids (Table 1).

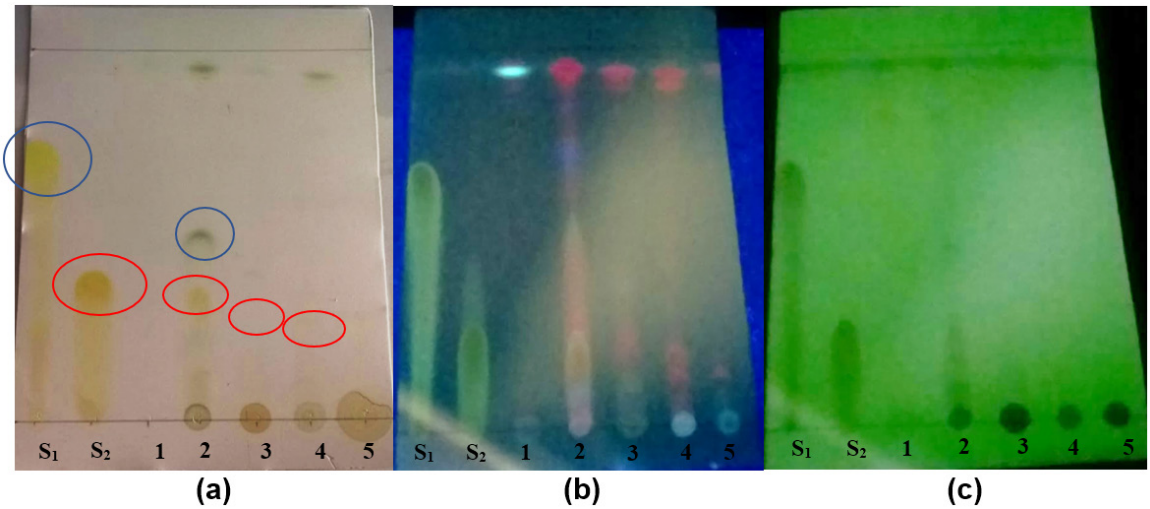


Figure 1. TLC of *Kleinhovia hospita* leaf extracts with quercetin and kaempferol standards: (a) after spraying with 5% $AlCl_3$, (b) under UV 366 nm, (c) under UV 254 nm. Stationary phase, silica gel 60 F_{254}; mobile phase, chloroform–ethyl acetate (5:2, v/v). S_1, kaempferol; S_2, quercetin; 1, n-hexane; 2, ethyl acetate; 3, 96% ethanol; 4, residual ethanol; 5, infusion. Circles are the original annotations (blue, kaempferol; red, quercetin)

Table 1. Phytochemical screening of the 96% ethanol extract and infusion of *Kleinhovia hospita* leaves

No.	Sample	Alkaloids	Flavonoids	Tannins	Saponins	Steroids	Triterpenoids
1.	96% ethanol	+	+	+	+	+	–
2.	Infusion	–	+	+	+	–	–

+, detected; –, not detected. Qualitative tube tests on a single preparation of each extract. Steroids and triterpenoids were read from the same Liebermann–Burchard test. Screening results for the leaf powder and the three sequential extracts were reported previously [2,3].

The infusion contained flavonoids, tannins, and saponins, but no alkaloids or steroids. Triterpenoids were not detected in either extract.

TLC identification of quercetin and kaempferol

After aluminium chloride spraying, the kaempferol and quercetin standards gave yellow spots at R_f 0.70 and 0.35, respectively (Figure 1). The ethyl acetate extract showed a yellow spot at R_f 0.32, which is consistent with quercetin. It also showed spots at R_f 0.10, 0.49, and 0.95, none of which matched the standards. The residual ethanol extract and the infusion showed only faint yellow zones near R_f 0.25, which were inconclusive. In the 96% ethanol extract, most yellow material remained near the origin, and no spots were visible in the n-hexane lane. No extract spot matched the kaempferol standard (R_f 0.70).

Quality parameters

The 96% ethanol extract had the highest ethanol-soluble extractive (35.87%), whereas the infusion had the highest water-soluble extractive (40.00%) (Table 2). The n-hexane extract contained no water-soluble matter. Specific gravity of the 5% dilutions ranged from 0.860 to 1.253.

Moisture content ranged from 3.37% to 9.42% in four extracts but reached 17.33% in the infusion, which exceeded the 10% limit (Table 2). Loss on drying followed the same pattern and was highest in the infusion (20.98%). Total ash was 5.57–5.97% in the ethyl acetate and ethanolic extracts and 8.92% in the infusion. Acid-insoluble ash was highest in the 96% ethanol extract (3.49%), about 63% of its total ash. All extracts met the microbial limits, with total plate counts of 3.6×10^2 to 1.2×10^3 CFU/g and mould and yeast counts of 2.7×10^2 to 5.8×10^2 CFU/g. Thus, four extracts met all regulated parameters, and the infusion failed only the moisture limit.

Discussion

This study yielded three main findings. Solvent polarity determined the yield, extractive values, and

Table 2. Specific and non-specific quality parameters of *Kleinhovia hospita* leaf extracts

Parameter	Limit	n-Hexane	Ethyl acetate	Residual ethanol	96% ethanol	Infusion
Specific parameters						
Ethanol-soluble extractive (%)	–	6.85	15.56	23.98	35.87	12.18
Water-soluble extractive (%)	–	0.00	9.93	20.00	15.05	40.00
Specific gravity of 5% dilution	–	0.870	0.860	1.031	1.253	1.224
Quercetin by TLC	–	−	+	±	−	±
Kaempferol by TLC	–	−	−	−	−	−
Non-specific parameters						
Loss on drying (%)	–	3.49	10.40	9.94	7.36	20.98
Moisture content (%)	≤10	3.37	9.42	9.03	6.83	**17.33**[a]
Total ash (%)	–	0.32	5.65	5.97	5.57	8.92
Acid-insoluble ash (%)	–	0.01	0.26	0.72	3.49	1.76
Total plate count (CFU/g)	$\leq 10^5$	3.6×10^2	8.9×10^2	7.7×10^2	5.2×10^2	1.2×10^3
Mould and yeast count (CFU/g)	$\leq 10^3$	3.0×10^2	2.7×10^2	4.5×10^2	3.9×10^2	5.8×10^2

Limits are from BPOM Regulation No. 32 of 2019 [14]; –, no applicable limit (value reported descriptively). [a]Exceeds the limit. Quercetin and kaempferol: +, spot matching the standard in R_f (±0.05) and colour after $AlCl_3$; ±, faint spot, inconclusive; −, not detected.
Values are single determinations of one preparation per extract; microbial counts are means of duplicate plates.
$AlCl_3$, aluminium chloride; CFU, colony-forming units; R_f, retardation factor; TLC, thin-layer chromatography.

phytochemical profile of tahongai leaf extracts. Four of the five extracts met the regulatory moisture and microbial limits, whereas the infusion exceeded the moisture limit. Re-examination of the TLC plates supported quercetin only in the ethyl acetate extract and did not confirm kaempferol in any extract.

The infusion gave a higher yield (44.61%) than single maceration with 96% ethanol (32.36%), because water dissolves polar constituents such as tannins, flavonoid glycosides, sugars, and mineral salts. Comparable solvent effects have been reported for other leaves, where methanolic and aqueous extracts gave higher yields than chloroform or ethyl acetate extracts [16,17]. The sequential extracts gave much lower yields (2.69–7.93%) in our previous report [3]. This difference reflects the lipophilic selectivity of n-hexane and the depletion of the marc by each preceding solvent.

The high yield of the infusion came at the cost of stability-related quality. Its moisture content (17.33%) and loss on drying (20.98%) were the highest, probably because freeze-dried aqueous extracts are rich in hygroscopic sugars and salts that take up water after drying. Its highest total ash (8.92%) is consistent with the extraction of water-soluble minerals. High moisture favours microbial growth and hydrolysis, so an infusion-based material would need controlled drying and moisture-proof packaging. Microbial contamination above regulatory limits is common in marketed herbal medicines [15,18]. In contrast, all extracts in this study remained well below the limits, although the infusion had the highest total plate count (1.2×10^3 CFU/g).

Our earlier standardization of a 70% ethanol extract of tahongai leaves from the same source reported a water content of 21.16%, total ash of 15.64%, and acid-insoluble ash of 8.28% [2]. The 96% ethanol extract in the present study had lower moisture (6.83%), total ash (5.57%), and acid-insoluble ash (3.49%), and a higher ethanol-soluble extractive (35.87% vs 18.30%) [2]. These differences probably reflect differences in harvesting, drying, and concentration. They show that extract specifications must be tied to a defined preparation process. Even so, acid-insoluble ash made up about 63% of the total ash of the 96% ethanol extract, which suggests residual siliceous matter such as soil. Better washing and sieving of the leaves before extraction should reduce this value.

The TLC results agree with the polarity of the solvents. Flavonoid aglycones such as quercetin are semi-polar and migrate in chloroform–ethyl acetate, so their detection in the ethyl acetate extract is expected. The 96% ethanol extract and infusion (Table 1), like

the residual ethanol extract in our earlier screening [3], were positive in the flavonoid tube test, yet their material stayed near the origin. This pattern suggests that their flavonoids occur mainly as polar glycosides, which this mobile phase cannot resolve. Our earlier report, based on a different plate with three extract lanes, stated that quercetin and kaempferol were present in the ethyl acetate and ethanol extracts, but it gave no R_f values [3]. On the present plate, the ethyl acetate spot at R_f 0.49, originally annotated as kaempferol, did not match the standard (R_f 0.70). Confirmation therefore requires a more polar mobile phase, co-spotting, and a quantitative method such as HPTLC densitometry or HPLC [13].

Taken together, the 96% ethanol extract combines a broad phytochemical profile, the highest ethanol-soluble extractive, and compliance with all regulated limits. Single maceration is also simple and gives a high yield, which makes this extract a practical candidate raw material. The ethyl acetate extract is the only one with a quercetin-consistent spot and may serve as a flavonoid-enriched fraction. However, its moisture content (9.42%) and loss on drying (10.40%) lie close to the 10% benchmark, so drying and storage conditions need control.

Several limitations affect the interpretation of these findings. Each extract was prepared once and most parameters were measured without replicates, so variability could not be estimated and no statistical comparison was possible. R_f values were read from plate photographs developed in a single, relatively non-polar mobile phase, and marker compounds were not quantified. Sequential and single-solvent extracts were obtained from different starting masses and depleted or fresh plant material, which limits direct comparison. Heavy metals, pesticide residues, and aflatoxins were not tested, and the leaves came from a single site and harvest. Future work should analyse at least three independent batches, quantify quercetin and total flavonoids by HPLC or HPTLC, and use a polar mobile phase to profile flavonoid glycosides. Stability studies of the 96% ethanol and ethyl acetate extracts are also needed before specifications can be proposed.

Conclusion

Solvent polarity shaped the yield, extractive values, and phytochemical profile of tahongai leaf extracts. All five extracts met the microbial limits, and four met the 10% moisture limit, whereas the infusion (17.33%) did not. The 96% ethanol extract combined the highest ethanol-soluble extractive (35.87%) with a broad phytochemical profile. The ethyl acetate extract was the only extract with a quercetin-consistent TLC spot. These two extracts are the most practical candidates for developing tahongai raw-material specifications, once replicate and quantitative marker analyses are available.

Acknowledgment

The authors thank the Herbarium of Andalas University (ANDA), Padang, for plant identification.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Author contributions

K: Conceptualization, Methodology, Investigation, Writing – original draft. IS: Investigation, Formal analysis, Writing – review & editing. H: Formal analysis, Writing – review & editing. All authors approved the final version of the manuscript.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Declaration of generative AI use

The authors did not use generative AI tools in preparing this manuscript.

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