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ANGIOTENSIN-CONVERTING ENZYME INHIBITORY, ANTIOXIDANT CAPACITY, PHENOLIC AND FLAVONOID CONTENT HERBONANOCEUTICAL OF TAMOENJU (*Hibiscus surattensis* L.)

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ABSTRACT

This research aimed to evaluate the herbonanoceutical effects of tamoenju leaf (*Hibiscus surattensis* L.) extract on Angiotensin Converting Enzyme (ACE) inhibition, antioxidant activity, total phenolic and flavonoid content. Tamoenju leaves were extracted through maceration with 96% ethanol. The extract was then formulated into nanoemulsions using VCO, the surfactants Tween 80 and Cremophor RH, and the cosurfactants propylene glycol and PEG 400. Antioxidant activity was assessed using DPPH and FRAP methods. ACE inhibition was tested with KIT-WST DOJINDO. Antioxidant activity for F1, F2, and F3 were 95.12 ± 3.55, 61.27 ± 3.84, and 160.40 ± 1.50 µg/mL (DPPH), and 51.92 ± 0.64, 135.60 ± 4.75, and 52.26 ± 2.22 µg/mL (FRAP), respectively. ACE inhibition (IC_{50}) values were 24.44 ± 0.81, 63.33 ± 0.10, and 52.28 ± 1.53 µg/mL for F1, F2, and F3. Phenolic content was highest in F3 (449.5 mg GAE/g), followed by F2 (383.03 mg GAE/g) and F1 (335.56 mg GAE/g). Flavonoid content was highest in F1 (328.77 mg QE/g), with F3 (300.91 mg QE/g) and F2 (240.43 mg QE/g) following. Formula F2 showed the highest antioxidant activity (DPPH), while F1 had the highest antioxidant activity (FRAP) and ACE inhibition, indicating F1's superior potential as an antioxidant and ACE inhibitor.

Keywords: Antioxidant, Flavonoid, Herbonanoceutical, *Hibiscus surattensis* L., Phenolic

RASĀYAN *J. Chem.*, Vol. 17, No. 4, 2024

INTRODUCTION

Herbonanoceuticals, which refer to herbal remedies reduced to nanosize particles, exhibit enhanced therapeutic efficacy and reduced adverse effects compared to contemporary pharmaceuticals.[1] Herbonanoceuticals have the potential to significantly benefit the field of therapeutics.[2] Dosage design influences drug delivery to receptors to produce the desired therapeutic effect. Nanotechnology systems continue to develop for active substances. that have problems with solubility, lipophilicity, permeability, and physiological degradation in the body. This is primarily due to nanostructured systems' potential to enhance the efficacy of plant extracts, minimize dosage requirements, mitigate adverse effects, and enhance overall activity.[3,4] The use of bioactive herbal components in producing plant-derived pharmaceuticals is prevalent. These compounds have unique properties that lead to lasting effects and good compatibility with the human body.[5] Alkaloids, tannins, phenols, flavonoids, tannins, sterols, and terpenes, are key phytochemicals with potential therapeutic benefits when taken in appropriate doses, making them promising candidates for use in herbal medicine.[6] Many naturally occurring compounds found in concentrates, including tannins, flavonoids, and terpenoids, are highly water-soluble. However, their absorption is limited due to challenges such as difficulty passing through lipid membranes, high molecular weights, and poor retention.[7] Using nano herbal formulations can enhance the targeted delivery of herbal medications, leading to improved selectivity, solubility, delivery, safety, and effectiveness.[8,9] Nanoscale pharmaceutical agents have an augmented surface area, facilitating expedited dissemination within the bloodstream and mitigating toxicity while preserving their therapeutic efficacy.[10] *Hibiscus surattensis* L., known locally as tamoenju in Central Sulawesi, is traditionally used to treat diabetes. Several previous studies have shown that tamoenju leaf extract acts as an antidiabetic by increasing insulin sensitivity, regenerating pancreatic β cells, increasing GLUT-4 expression, and inhibiting the DPP-4 enzyme

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inhibitor.[11,12] Tamoenju leaf extract also acts as an angiotensin-converting enzyme (ACE) inhibitor.[13] Providing antioxidants is an effort to inhibit the production of free radicals or increase the ability of defense enzymes against free radicals to prevent oxidative stress and vascular complications related to diabetes.[14,15] The potential of tamoenju leaf extract is limited by the low solubility and bioavailability of the active substance via the oral route. According to previous research, the stability of the active substance can be maintained through the formation of nanoemulsion preparations. The development of herbonanoceutical tamoenju leaf extract aims to increase bioavailability, solubility, absorption of active substances, and pharmacological effects, especially antioxidants and ACE inhibitors, so it can be used as adjuvant therapy for complications of diabetes mellitus.

EXPERIMENTAL

Material and Methods

The ingredients used are olive oil, Virgin coconut oil (VCO), propylene glycol, Tween 80, Cremophor RH 40, polyethylene glycol (PEG) 400, and ethanol 96% from Brataco, Indonesia, aquadest (Waterone). The chemicals DPPH, TPTZ (2,4,6-tripyridyl-S-triazine), quercetin, gallic acid, ascorbic acid, aluminum chloride, sodium acetate, and NaOH were obtained from Sigma Aldrich. Reagent Folin-Ciocalteu (Merck, Germany). The ACE kit-WST by PT. Genetika Science Indonesia, Captopril (Infalabs BPOM). In this study, we employed analytical tools including glassware (Pyrex®), micropipette (Biologix), incubator (Memmert®), pH meter (LAQUA PH1100), centrifuge (DLab D3024), UV-visible spectrophotometer (Cecil-CE7410), analytical balance (Sartorius), and vortex (Labnet VX-200).

General Procedure

The source of the tamoenju leaf was Alindau village in Sindue Tobata District, Central Sulawesi Province's Donggala Regency. Plants were identified in the Plant Systematics Laboratory, Biology Department, FMIPA, Tadulako University, Palu City, Central Sulawesi with No.031/UN28.1.28/BIO/2023. The simplicia was made, including dry sorting, washing, wet sorting, chopping, and drying. The simplicia was ground into powder and stored in a dry, tightly sealed container for use in the subsequent research stage.

Extract Preparation

Tamoenju leaf extract was prepared using the maceration method with 96% ethanol as the solvent. The maceration process lasted for five days, with the solvent being replaced every 24 hours and stirred continuously every six hours. The resulting maserate was concentrated using a rotary evaporator to remove most of the solvent, and then it was further evaporated over a water bath to produce a thick extract. The extract yield obtained was 21.88%.

Manufacture of Herbonanoceuticals of Tamoenju Leaf Extract

100 mg of tamoenju leaf extract was added to 100 ml of a mixture of oil, surfactant, and cosurfactant, homogenized with a magnetic stirrer for 30 minutes then ultrasonicated with an amplitude of 40% for 20 minutes.

Antioxidant Test Using DPPH Methods

The test method was carried out according to previous research with slight modifications.[16] The concentration of the sample stock solution was varied (25, 50, 100, and 200 µg/mL). The comparison solution used was ascorbic acid (2.5, 5, 10, 20, 40, and 60 µg/mL). The following formula was used to determine the capacity to scavenge the DPPH radical.

$$\text{DPPH scavenged (\%)} = \frac{(x-y)}{x} \times 100 \qquad (1)$$

Where x is absorbance of blank; y is absorbance of the sample. Calculating the inhibitory concentration of 50% (IC_{50}) was done using GraphPad Prism 8.0 software.

Ferric Reducing Antioxidant Power

Determination of antioxidant activity using the FRAP test with ascorbic acid as standard using the previously described methodology.[17] The concentration of the sample stock solution was varied (25, 50, 100, 200, and 400 µg/mL). The comparison solution used was ascorbic acid (2, 4, 6, 8, and 10 µg/mL). Measurement of ascorbic acid absorption was carried out using the same method as the sample measurement. All the determinations were performed in triplicate. EC_{50} of FRAP capacity of each formula can be calculated using its calibration curve.

$$Abs = - \log Ts \quad (2)$$

$$\% \text{ Antioxidant capasity} = (1 - Ts) \times 100\% \quad (3)$$

Abs = Absorbance
Ts = Transmittance
The antioxidant capacity percentage was plotted against the concentration, and the EC_{50} value was calculated using Graph Prism 8.0 software.

ACE Inhibitory Activity Determination
The activity of angiotensin-converting enzyme inhibition was assessed using the ACE inhibition assay kit (ACE kit-WST).[18] The working solution was prepared according to the kit's instruction booklet. Absorbance was measured at 450 nm using a microplate reader. The ACE inhibitory activity was then calculated using the following formula:

$$\text{ACE inhibitory activity} = \frac{(Ac - As)}{(Ac - As)} \times 100 \quad (4)$$

As: absorbance of wells treated with the sample
Ac: absorbance of control wells
Ab: absorbance of blank wells
IC_{50} was determined using Graph Pad 8.0 Prism. The standard solutions were tested by captopril solutions (2, 4, 8, 16, 32, and 64 µg/mL) and herbonanoceutical formulas 12.5, 25, 50, 100, 200, and 400 µg/mL.

Determination of Total Phenolic Content
The total phenol content was measured using the Folin-Ciocalteu reagent, with gallic acid serving as the standard.[19] A standard calibration curve was created by preparing a gallic acid stock solution at a concentration of 500 µg/mL, which was subsequently diluted to achieve concentrations of 10, 20, 40, 80, 120, and 160 µg/mL. Using the absorbance measurements, total phenol content was calculated with the linear regression equation from the gallic acid standard curve and expressed as gallic acid equivalent per gram (mg GAE/g).

Determination of Total Flavonoid Content
The total flavonoid content was determined using quercetin as the standard. A stock solution of quercetin was prepared by dissolving it in analytical-grade ethanol at a concentration of 500 µg/mL. Serial dilutions were then made to achieve concentrations of 20, 40, 60, 80, and 100 µg/mL. This procedure was repeated three times. The total flavonoid content was calculated using the linear regression equation derived from the quercetin standard curve and expressed as quercetin equivalent per gram (mg QE/g).[20]

RESULTS AND DISCUSSION

Preparation of Herbonanoceutical
The extract obtained was made into a nanoemulsion preparation using the VCO oil phase, surfactant, and cosurfactant, specifically Tween 80-propylene glycol, and Cremophor RH 40-PEG 400, according to optimization results from previous research. In previous research, three optimum formulas were obtained with a ratio of oil phase to surfactant and cosurfactant of 1:9, while the optimum ratio of surfactant to cosurfactant was formula F1 Tween 80: propylene glycol (2:1), formula F2 (3:1); and formula F3 Cremophor RH 40: PEG 400 (1:1). The optimum herbonanoceutical formula contains 0.1% tamoenju leaf extract as shown in Fig.-1.

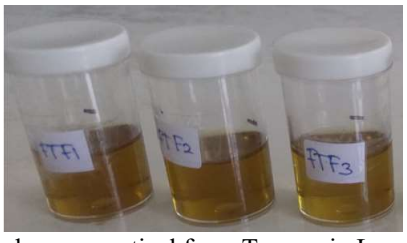
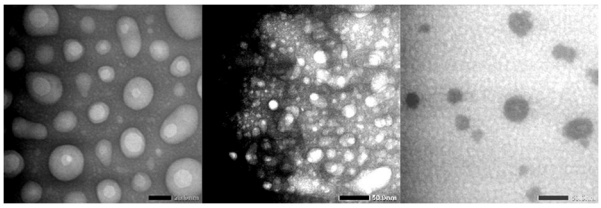


Fig.-1: Herbonanoceutical from Tamoenju Leaves Extract

Morphological observations herbonanoceutical of tamoenju leaf extract using TEM can be seen in Fig.-2.



(a) (b) (c)

Fig.-2: Particle Morphology (a) Formula F1 at a Magnification of 150.000 times (b) Formula F2 at a Magnification of 80.000 times (c) Formula F3 at a Magnification of 80.000 times Using Transmission Electron Microscopy (TEM)

Figure-2 shows F1 has particles with a homogeneous size distribution. This indicates that the formulation used is effective in producing particles with uniform sizes. F2 shows a greater variation in particle size compared to F1. This variation may be due to differences in the formulation process, or materials used. F3 has a more heterogeneous particle size distribution with some larger particles scattered around. This indicates that the formulation process for F3 produces particles with varying sizes. The F2 formula has an irregular shape, indicating agglomeration and uneven distribution. In contrast, the F1 and F3 formulas show spherical nanoparticles. Morphology is crucial because non-spherical shapes can easily encounter other nanoparticles, leading to easy aggregation of the preparation.[21]

Antioxidant Activity Evaluation of Herbonanoceutical Formulas using DPPH and FRAP Methods

The antioxidant activity of the herbonanoceutical formula was evaluated using the DPPH and FRAP methods. Figure-3 displays the IC_{50} values for both the herbonanoceutical formula and ascorbic acid as a standard. Although the IC_{50} value of ascorbic acid was lower than that of the herbonanoceutical formula, the IC_{50} values for ascorbic acid, F1, and F2 were in the same category of powerful antioxidants, except for F3, which is in the medium category.[22] Antioxidant activity can also be based on the value of AAI (Antioxidant Activity Index). The AAI values are as follows: ascorbic acid 29.63, F1 4.21, F2 6.53, and F3 2.49, all classified as very strong.[23] Meanwhile, in previous research, the AAI value of the tamoenju leaf extract was classified as moderate.[11]

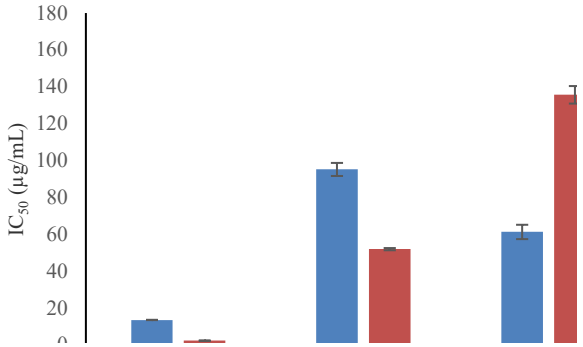


Fig.-3: Herbonanoceutical Antioxidant Activity of Tamoenju Leaf Extract using DPPH and FRAP Methods. The Data are Shown as Mean ± Standard Deviation, with n=3

Antioxidant testing was also conducted using the FRAP method. In the FRAP method testing, F1 showed a better reduction capacity compared to antioxidant activity based on the DPPH test. F2 showed a lower reduction capacity compared to antioxidant activity based on the DPPH test. F2 is likely more effective in scavenging free radicals than in reducing metal ions. Meanwhile, F3 showed the opposite result to F2, where the reduction capacity was higher than the antioxidant activity, indicating a very good reduction ability but low antioxidant activity. These results indicate that each sample has different characteristics in terms of antioxidant activity and reduction capacity, which can be influenced by the chemical composition and working mechanism of the compounds in each sample. The differences between the DPPH and FRAP measurement results for antioxidant capacity are due to not all antioxidant components functioning as reducing agents and radical scavengers simultaneously.[24] The DPPH and FRAP procedures have different reaction processes, with DPPH using an electron transfer mechanism and FRAP using a reduction mechanism.[25] The process of nano-emulsification can explain the improved antioxidant activity of tamoenju

leaf extract. Due to the reduction in droplet size, the surface area inside the nanodroplets is increased, making the antioxidant compounds more accessible to interact with the DPPH and FRAP free radicals.[26] It is crucial to note that the tamoenju leaf extract will disperse more readily after the nano-emulsification process, which will greatly enhance oral absorption when applied in vivo. The final research results showed that tamoenju leaf extract formulated in herbonanoceutical form increased antioxidant power compared to the extract form. The increase in antioxidant activity in nanoemulsions can be attributed to the solubility of the extract in small granules in the nanometer range, which increases the surface area and accelerates contact with free radicals. This makes the herbonanoceutical more effective in fighting free radicals from DPPH.

Evaluation of Herbonanoceutical of Tamoenju Leaf Extract as an ACE Inhibitor

The ACE inhibitory activity of herbonanoceutical extract tamoenju leaves is presented in Table-1.

Table-1: ACE Inhibitory Activity of Herbonanoceutical from Tamoenju Leaves Extract

No	Sample	IC_{50} (µg/mL)
1	Captopril	1.69 ± 0.61
2	F1	24.44 ± 0.81
3	F2	63.33 ± 0.10
4	F3	52.28 ± 1.53

The data are presented as mean ± standard deviation, with n=3

Table-1 shows that F1, F2, and F3 can be used for antihypertensive therapy because their IC_{50} values fall into the very strong (F1) to strong (F2 and F3) categories. Captopril has the lowest IC_{50} value of 1.69 ± 0.61 µg/mL, indicating it is the most potent ACE inhibitor among the samples tested. F1 demonstrates the greatest potential as an ACE inhibitor among the tamoenju leaf extract formulations. The data indicates that F1 could be a promising candidate for further development as an ACE inhibitory agent. In previous research, the IC_{50} of tamoenju leaf extract as an ACE inhibitor was 38.50 ± 1.41 µg/mL.[13] The results obtained indicate that herbonanoceutical tamoenju leaf extract has activity as an ACE inhibitor. This is associated with the ability of nanoemulsion to diffuse into body cells better than non-nanoemulsion preparations, which have a larger size and thus inhibit the entry of active substances into cells.[27] However, this data is still limited, and further studies are necessary. In vivo, studies need to be conducted to confirm the antihypertensive activity and the antioxidant capacity of the herbonanoceutical tamoenju leaf extract.

Total Phenolic and Flavonoid Content Herbonanoceutical of Tamonju Leaf Extract

The concentration data for phenolics and flavonoids from each formula are presented in Table-2.

Table-2: Total Phenolic and Flavonoid Content of Herbonanoceutical from Tamoenju Leaves Extract

No	Sample	Total phenolic content (mgGAE/gram)	Total flavonoid content (mgQE/gram)
1	F1	335.56±5.68	328.77±17.91
2	F2	383.02±6.5	240.43±9.57
3	F3	449.50±7.62	300.91±15.77

The data are presented as mean ± standard deviation, with n=3

Table-2 shows the results of the measurement of the test sample for determining the total phenolic and flavonoid content made as many as 3 replications. The total phenolic content showed the highest value in F3 (449.50 ± 7.62 mg GAE/gram) and the lowest in F1 (335.56 ± 5.68 mg GAE/gram). The total flavonoid content showed the highest value in F1 (328.77 ± 17.91 mg QE/gram) and the lowest in F2 (240.43 ± 9.57 mg QE/gram). The total phenolic and total flavonoid content in a sample greatly affects the antioxidant activity and ACE inhibitor produced. Based on the analysis results obtained, a correlation test of bioactive compounds against antioxidants and ACE inhibitors was then carried out using the Pearson correlation test which is shown in Table-3.

Based on the correlation results between total phenolic and flavonoid contents with IC_{50} DPPH, FRAP, and ACE inhibitor in Table- 3, it is shown that total phenolic content has a significant negative correlation with IC_{50} DPPH antioxidant activity.

This indicates that the higher the phenolic content, the lower the IC_{50} DPPH, meaning that antioxidant activity increases. Total flavonoid content shows a significant negative correlation with EC_{50} FRAP and

IC_{50} ACEI, indicating that the higher the flavonoid content, the lower the IC_{50} for these two parameters, which means increased antioxidant activity and ACE inhibition. Total flavonoid content has an insignificant positive correlation with IC_{50} DPPH, indicating that the relationship between flavonoid content and IC_{50} DPPH antioxidant activity is not significant in this study.

Table-3: Correlation of Total Flavonoid and Phenolic Content with IC_{50} and EC_{50}

No	Parameter	Pearson's Correlation Coefficient (r)	
		Total Phenolic	Total Flavonoid
1	IC_{50} DPPH	-0.814**	0.347
2	EC_{50} FRAP	-0.0890	-0.896**
3	IC_{50} ACEI	0.620	-0.846**

**. Correlation is significant at the 0.01 level (2-tailed)

This correlation results could explain that the highest antioxidant activity and ACE inhibitor was given by herbonanoceutical from tamoenju leaf extract which had the highest flavonoid content and lowest phenolic content therefore flavonoid compounds were the major contributors of antioxidant activity and ACE inhibitor. These results are supported by previous research, which found that tamoenju leaf extract contains at least five flavonoid compounds: quercetin, kaempferol, morin, trifolin, and rhamnazin.[11]

CONCLUSION

Formula F1 herbonanoceutical of tamoenju leaf extract has the greatest potential as both an antioxidant agent and an ACE inhibitor. The total flavonoid content plays the most dominant role in producing antioxidant activity and angiotensin-converting enzyme inhibition in the herbonanoceutical extract of tamoenju leaves. However, further studies, including in vivo evaluations, are necessary to confirm these findings and explore their clinical relevance.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

AUTHOR CONTRIBUTIONS

All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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