In vitro evaluation of nanoemulsion formulations of *Orthosiphon aristatus* (Blume) Miq. and *Persea americana* Mill. extracts as anti-urolithiasis agents

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Abstract. Urolithiasis is a condition characterized by the formation of stones in the urinary tract due to crystal deposits. The disease has a high prevalence in various countries. Efforts to prevent urolithiasis using conventional therapy are still limited. The use of a combination of traditional medicinal plants, *Orthosiphon aristatus* (Blume) Miq. and *Persea americana* Mill., which contain quercetin, sinensetin, and rosmarinic acid, has emerged as an alternative treatment due to their potential as anti-urolithiasis agents that can support efforts to prevent stone formation. A major challenge in utilizing these two plants is harnessing their bioactive compounds, which are poorly water-soluble and have limited bioavailability. The aim of this study is to formulate a stable nanoemulsion. Nanoemulsion formulations were developed to improve solubility and bioavailability using the SNEDDS method with extract ratios of 1:4 and 4:1, and in vitro drug release tests were performed. The solubility tests showed that the best solvents were Tween 80, Span 80, and IPM, and the pseudo-ternary phase diagram showed a 1:1 ratio. Characterization and in vitro results indicated stability and rapid, substantial release of active compounds. This study demonstrates that the formulation combining both plants is effective in providing anti-urolithiasis activity.

1 Introduction

Urolithiasis is a condition characterized by the formation of stones due to the deposition of crystals in the urinary tract. Crystal deposition begins in the kidneys, ureters, bladder, and urethra [1]. The crystals that have deposited into stones are generally composed of calcium oxalate, calcium phosphate, uric acid, magnesium-ammonium-phosphate (MAP), xanthine, cystine, and several other compounds. Identifying stone composition is important for preventing recurrent stones [2]. The prevalence of urolithiasis varies across countries; in the United States, about 5-10% of the population experiences this condition, with around 7% in

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adult women and 13% in adult men. In Indonesia, urolithiasis ranks among the top three most common urological conditions, along with urinary tract disorders [3]. The pathogenesis of urolithiasis in humans and animals is generally similar, starting with the triggering of calcium and oxalate supersaturation, which increases crystallization-promoting factors and decreases crystallization-inhibiting factors [4].

The high prevalence and complexity serve as a reference for the mechanism of stone formation. The management of urolithiasis must be given serious attention. Efforts to prevent urolithiasis continue to this day. Prevention measures for urolithiasis are still largely surgical and invasive, limiting their effectiveness. The formation process of calcium oxalate stones involves complicated stages, starting with nucleation or the formation of crystal nuclei in the urine. Nucleation occurs either homogeneously (between similar ions) or heterogeneously (triggered by foreign particles). The crystals will undergo growth and agglomeration (the joining of smaller crystals into larger masses). This process occurs via a free-particle mechanism in the urine or a fixed-particle mechanism, in which crystals attach to the epithelium of the urinary tract [5].

The limitations of conventional therapy and the high risk of recurrence have driven the discovery of safer, more accessible alternatives, including the use of traditional plants. *Persea americana* Mill. and *Orthosiphon aristatus* (Blume) Miq. have long been empirically used by communities to manage hypertension and urinary tract disorders. The use of these traditional plants is supported by scientific evidence showing that previous studies indicate that the combination of these two plants has anti-urolithiasis, diuretic, and antioxidant activities, effective in inhibiting stone formation [6].

The effectiveness of the combination of these two plants is supported by the levels of their secondary metabolites. *Persea americana* Mill. contains quercetin, a flavonoid compound with antioxidant activity that has been widely reported to be better than other flavonoid compounds [7]. *Orthosiphon aristatus* (Blume) Miq. contains important compounds, sinensetin and rosmarinic acid. Sinensetin is a phenolic compound with proven antioxidant activity in the body [8].

The use of traditional medicinal plants, when combined, poses a major challenge for harnessing their bioactive compounds. The bioactive compounds utilized have low water solubility and limited bioavailability. Their low water solubility and limited bioavailability result in suboptimal absorption, requiring higher doses. To address these challenges, nanoemulsion-based formulations have been developed. Nanoemulsion-based preparations are oil-in-water dispersion systems at the nanometer scale. The development of nanoemulsion-based formulations can improve solubility and stability, enhance the bioavailability of hydrophobic active ingredients, reduce dosage requirements, protect active ingredients from enzymatic degradation, and increase the absorption of active substances. The combination of avocado leaf extract and cat's whiskers is expected to optimize its pharmacological effects and work more effectively and efficiently in the prevention and therapy of urolithiasis.

2 Material and methods

2.1 Material

The plant species of *Persea americana* Mill. and *Orthosiphon aristatus* (Blume) Miq. were obtained from BALITRO Bogor. Artificial urine was obtained from the Veterinary Pharmacy Laboratory of the Faculty of Veterinary Medicine and Biomedical Sciences, IPB University. Calcium oxalate stones were obtained from the Veterinary Pathology Laboratory of the Faculty of Veterinary Medicine and Biomedical Sciences, IPB University. This study used a

combination of plant extracts of *Persea americana* Mill. and *Orthosiphon aristatus* (Blume) Miq. in ratios of 1:4 and 4:1. Nanoemulsion components include surfactants (Tween 20, Tween 80, Span 80), co-surfactants (PEG 400, PG), and solvents (Glycerin, VCO, IPM). Distilled water was used as the aqueous phase. 70% ethanol was used as the extraction solvent. Gallic acid and quercetin were used as reference standards for total phenolic and total flavonoid content analysis. A dialysis bag was used as the medium for in vitro drug release testing.

2.2 Preparation of extracts of *Persea americana* Mill. and *Orthosiphon aristatus* (Blume) Miq.

The extract was prepared by maceration with 70% ethanol. The medicinal plants *Persea americana* Mill. and *Orthosiphon aristatus* (Blume) Miq. were extracted separately with a solvent-to-material ratio of 1:10 (w/v). The extracts were macerated for 72 hours at room temperature. The maceration filtrate was filtered to obtain a clear filtrate. Evaporation was carried out using a rotary evaporator at 40-50°C until a thick extract was obtained.

2.3 Solubility test

The solubility of *Persea americana* Mill. and *Orthosiphon aristatus* (Blume) Miq. Extracts were tested using several surfactant solvents, co-surfactants, and solvents. 1 gram of extract was mixed with 3 ml of each carrier. The extracts mixed with each carrier were homogenized at 200 rpm for 48 hours. The homogenized extract and carrier were centrifuged at 6000 rpm for 15 minutes. The resulting supernatant was filtered, and the filtrate was analyzed for total phenolic content using a UV-Vis spectrophotometer to determine the highest solubility [9].

2.4 Pseudo-ternary phase diagram

The selected surfactants, Tween 80 and Span 80, were mixed together until homogeneous using an Sblend ratio of 7:3. Smix was mixed with co-surfactants using Smix ratios of 1:1, 1:2, and 2:1. The mixed surfactants and co-surfactants were homogenized until a clear solution was formed. The mixed Smix was then combined with the solvent using ratios of 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, and 9:1 (w/w). Titration was carried out with distilled water until turbidity appeared, indicating the nanoemulsion boundary. The amount of distilled water titrated was recorded and plotted using Triplot software to obtain a pseudo-ternary phase diagram for mapping the nanoemulsion region.

2.5 Nanoemulsion formulation

Based on the results of the solvent and the pseudo-ternary phase diagram, the best solvent was mixed with the extract at ratios of 1:4 and 4:1. Each combined sample, weighing 3 grams, was then mixed with Smix 1:1 and IPM in ratios from 1:9 to 9:1 (w/w). Mixtures were adjusted to reach a total of 30 grams. They were homogenized and sonicated until clear. The resulting mixture was diluted 100 times.

2.6 Characterization of nanoemulsions

Nanoemulsion characterization is used to ensure that the resulting formulation is stable and to determine the suitability of the average droplet size (Z-average). Measurements are carried out using zeta potential with a Zetasizer Nano ZS, while particle size analysis (PSA) is

performed using the Dynamic Light Scattering (DLS) method with a Malvern Zetasizer Nano ZS. The diluted samples are placed in a quartz cuvette, and measurements are initiated at 25°C. Each sample is analyzed in triplicate to obtain the mean value and standard deviation.

2.7 *In vitro drug release*

In vitro drug release testing was performed using the dialysis bag method with phosphate-buffered saline (PBS) at pH 7.4. 5 ml of the best formulation was placed into the dialysis bag and immersed in 20 ml of phosphate-buffered saline (PBS) as the release medium. Continuous stirring was performed at 100 rpm at room temperature. At predetermined times, samples were collected from the phosphate-buffered saline (PBS) medium and analyzed for flavonoid content using the aluminum chloride colorimetric method with quercetin as the reference standard.

3 Result and discussion

3.1 Solubility test

Solubility testing was conducted to observe the solubility of extracts from *Orthosiphon aristatus* (Blume) Miq. and *Persea americana* Mill.. The solubility test was carried out using ratios of 1:1, 1:4, and 4:1, with the addition of respective surfactants (Tween 80, Tween 20, Span 80), co-surfactants (PEG 400, PG), and solvents (IPM, VCO, Glycerin). The results of the solubility test are shown in Table 1.

Table 1. Solubility profile of *Orthosiphon aristatus* and *Persea americana* extracts in various surfactants, co-surfactants, and solvents.

Treatment	Surfactant			Co-surfactant			Solvent	
	Tween 80	**Tween 20**	**Span 80**	**PEG 400**	**PG**	**IPM**	**VCO**	**Gliserin**
Control	-	-	-	-	-	-	-	-
1g Pa (1:1)	+++	++	+	+++	+++	+	++	+
1g Oa (1:1)	+++	+++	+++	+++	+++	+++	++	+++
0,2g Pa + 0,8g Oa (1:4)	+++	+++	+++	+++	+++	+++	++	+++
0,8g Pa + 0,2g Oa (4:1)	+++	+++	+++	+++	+++	+++	++	+++

Description: + : insoluble, ++ : moderately soluble, +++: soluble

Based on the solubility test results, each extract treatment exhibited different degrees of solubility with each surfactant, co-surfactant, and solvent. The comparison indicated that the best solubility and the most stable absorbance curve, with a clear absorption peak, were achieved with the surfactants (Tween 80 and Span 80), the co-surfactant (PG), and the solvent (IPM). The absorbance curve and solubility results showed that the surfactants (Tween 80 and Span 80) dissolve better and are more stable compared to the surfactant (Tween 20). The

absorbance curve and solubility of the co-surfactant (PG) were better and more stable compared to the co-surfactant (PEG 400). The absorbance curve and solvent capability (IPM) were better and more stable than those of the solvents (VCO and Glycerin).

A solvent that dissolves well is one that dissolves the extract at the right concentration and ratio without precipitation, and must be selected. In several studies, Tween 80 has been shown to be an effective solvent for dissolving herbal extracts. Other studies have also confirmed that the combination of Tween 80, PG, and PEG 400 can increase solubility without significantly affecting viability [10]. The selected best solvent is used to prepare a ternary diagram to determine which solvent formulation yields the best nanoemulsion mapping.

3.2 Pseudo-ternary phase diagram

A pseudo-ternary phase diagram was created to map the most stable regions of the nanoemulsion system and its three main components. Tests to make the pseudo-ternary phase diagram used ratios of 1:1, 1:2, and 2:1. The results of the nanoemulsion region mapping are shown in the diagram and Table 2.

Table 2. Composition ratios and percentage distribution of smix, oil, and water in the pseudo-ternary phase diagram for nanoemulsion mapping.

Trial ID	Sblend	Smix	Smix:Oil	Smix (g)	Oil (g)	Smix (%)	Oil (%)	Water (%)
T001	7:3	1:1	1:9	10	90	9.46	85.18	5.36
T002	7:3	1:1	2:8	20	80	18.72	74.86	6.42
T003	7:3	1:1	3:7	30	70	27.65	64.52	7.83
T004	7:3	1:1	4:6	40	60	35.43	53.14	11.43
T005	7:3	1:1	5:5	50	50	43.01	43.01	13.99
T006	7:3	1:1	6:4	60	40	53.09	35.39	11.52
T007	7:3	1:1	7:3	70	30	62.31	26.70	10.98
T008	7:3	1:1	8:2	80	20	66.21	16.55	17.23
T009	7:3	1:1	9:1	90	10	49.21	5.47	45.32
T010	7:3	1:2	1:9	10	90	52.93	44.80	2.27
T011	7:3	1:2	2:8	20	80	52.41	44.36	3.23
T012	7:3	1:2	3:7	30	70	52.86	44.74	2.40
T013	7:3	1:2	4:6	40	60	53.21	45.04	1.76
T014	7:3	1:2	5:5	50	50	53.17	45.01	1.82
T015	7:3	1:2	6:4	60	40	53.63	45.40	0.97

Table 2. Composition ratios and percentage distribution of smix, oil, and water in the pseudo-ternary phase diagram for nanoemulsion mapping (continue).

Trial ID	Sblend	Smix	Smix:Oil	Smix (g)	Oil (g)	Smix (%)	Oil (%)	Water (%)
T016	7:3	1:2	7:3	70	30	53.79	45.53	0.68
T017	7:3	1:2	8:2	80	20	53.39	45.19	1.42
T018	7:3	1:2	9:1	90	10	52.78	44.67	2.55
T019	7:3	2:1	1:9	10	90	52.01	44.02	3.97
T020	7:3	2:1	2:8	20	80	51.52	43.61	4.88
T021	7:3	2:1	3:7	30	70	52.13	44.12	3.74
T022	7:3	2:1	4:6	40	60	52.86	44.74	2.40
T023	7:3	2:1	5:5	50	50	52.81	44.70	2.49
T024	7:3	2:1	6:4	60	40	52.87	44.75	2.38
T025	7:3	2:1	7:3	70	30	52.47	44.41	3.12
T026	7:3	2:1	8:2	80	20	49.39	41.80	8.81
T027	7:3	2:1	9:1	90	10	52.36	44.32	3.33

Description: Sblend = (Tween80 : Span80), Smix = (Sblend:PG), Smix to Oil = ((Sblend:PG) : IPM))

Based on the results in Table 2, the pseudo-ternary test at a smix ratio of 1:1 (T001-T009) yielded the best results, with smix values ranging from 9.46% to 62.31%, oil values ranging from 5.47% to 85.18%, and water values ranging from 5.36% to 45.32%. The formulation at a 1:1 ratio provides a balanced distribution value. The resulting values indicate the formation of a fairly stable nanoemulsion region due to the influence of surfactant and co-surfactant being at their maximum efficiency points. This ratio also represents the optimal condition for spontaneous nanoemulsion formation during titration.

Based on Table 2, the pseudo-ternary ratio smix test at 1:2 (T010-T018) showed that the smix values ranged from 32.01% to 53.79%, oil values ranged from 14.80% to 46.83%, and water values ranged from 0.97% to 2.27%. The range of values obtained indicates this because the variation between experiments was quite large. These values demonstrate that increasing the concentration of the co-surfactant (PG) leads to an imbalance in surfactant molecules, rendering the emulsion unstable.

Based on the results in Table 2 for the pseudo-ternary test of the smix ratio at 2:1 (T019-T027), the 2:1 ratio was the least stable, with smix values ranging from 49.39% to 52.87%. The oil values ranged from 41.50% to 44.75%, and the water values ranged from 2.18% to 8.31%. The obtained values indicate that the surfactant is not working optimally.

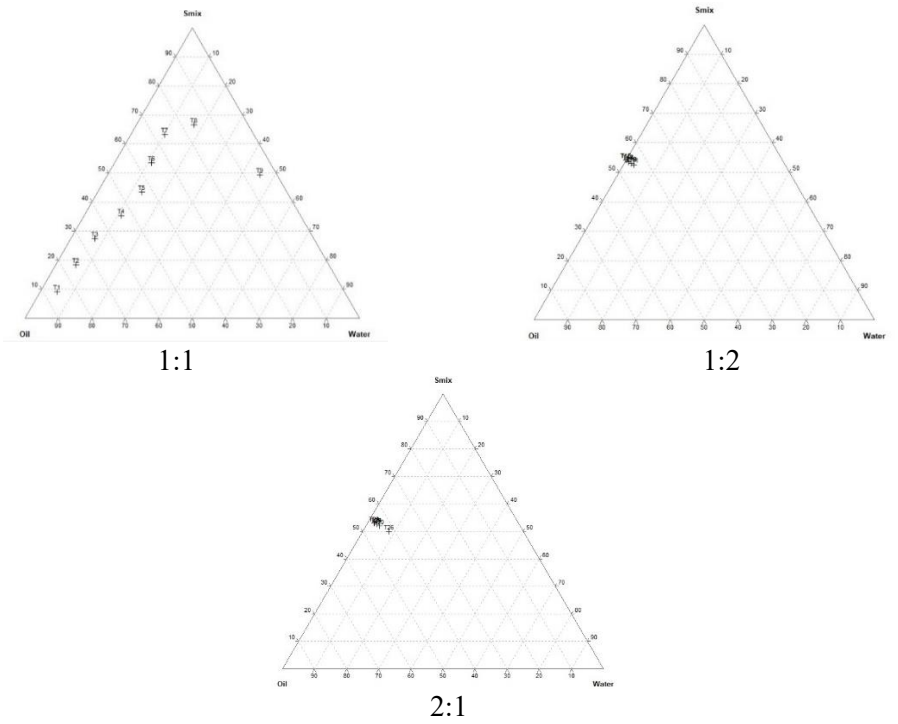

Fig. 1. Pseudo-ternary phase diagram of nanoemulsion system at smix ratios of 1:1, 1:2, and 2:1.

Based on Figure 5 in the pseudo-ternary diagram, the mapping point for the nanoemulsion region is identified at a 1:1 ratio. The dispersion point is well-defined, indicating that the nanoemulsion phase forms within the wider shaded area. The 1:1 ratio signifies that the formulation used shows the formation of the nanoemulsion region.

At the 1:2 ratio, the mapping points for the nanoemulsion region are more concentrated in the Smix area, while there are fewer points in the water region. The instability observed may be triggered by the high oil usage.

At the 2:1 ratio, the points are mostly in the Smix region, indicating that increasing the surfactant does not create a large nanoemulsion region and cannot produce good consistency. The nanoemulsion's ability to map a stable region is influenced by the balance between interfacial tension reduction and flexibility.

Surfactants and co-surfactants combined at a 7:3 ratio produce a more effective HLB for nanoemulsion mapping. In a 1:1 smix, surfactant, co-surfactant, and solvent can work optimally. The surfactant fraction can reduce tension, while the co-surfactant can increase fluidity, thereby producing a large nanoemulsion area [11].

3.3 Nanoemulsion formulation

Nanoemulsion formulation as the determination of the best optimum nanoemulsion by mixing *Orthosiphon aristatus* (Blume) Miq. and *Persea americana* Mill. into a selected surfactant, co-surfactant, and solvent mixture. The observation results are shown in Table 3, with a 1:1 ratio, to determine whether phase separation occurs in the nanoemulsion sample.

Based on Table 3, the nanoemulsion formulations with an extract ratio of 1:4 and Smix:solvent ratios of 1:9 and 2:8 showed phase separation, whereas formulations with ratios of 3:7, 4:6, 5:5, 6:4, and 7:3 did not. At an extract ratio of 4:1 with a Smix:oil ratio of 1:9, 2:8, 3:7, 4:6, 5:5, and 6:4, phase separation occurred, whereas at ratios of 7:3, 8:2, and 9:1,

no separation was observed. The occurrence of phase separation indicates instability or that the formulation used is not yet optimal.

Table 3. Phase separation observation of nanoemulsion formulations at different extract ratios and smix:oil ratios (1:1 system).

Extract Ratio	Smix : Oil Ratio	Separation Phase	
		Exist	Not
1:4	1:9	✓	
	2:8	✓	
	3:7		✓
	4:6		✓
	5:5		✓
	6:4		✓
	7:3		✓
	8:2		✓
	9:1		✓
4:1	1:9	✓	
	2:8	✓	
	3:7	✓	
	4:6	✓	
	5:5	✓	
	6:4	✓	
	7:3		✓
	8:2		✓
	9:1		✓

Note: ✓= Occurrence of separation

3.4 Characterization of nanoemulsions

3.4.1 Zeta Potential and Particle Size Analyzer (PSA)

Based on the results in Table 4 of the nanoemulsion characterization using zeta potential and particle size analyzer (PSA), the best results were obtained with an extract ratio of 1:4 in samples F7, F8, and F9. The nanoemulsion formulations that underwent characterization had an average particle size (Z-average) of 1:4, F7 (51.15 nm), F8 (148.2 nm), F9 (101.6 nm), and 4:1, F8 (68.52 nm), F9 (165.2 nm), with a fairly good size distribution, indicating that the system was fully homogeneous.

A lower solvent ratio combined with a relatively higher surfactant concentration will more effectively reduce interfacial tension, promoting the formation of finer nano droplets and a clearer, more transparent emulsion. Conversely, a higher solvent-to-surfactant ratio results in larger droplets and a cloudier emulsion due to unstable emulsification. In some cases, a higher solvent content can also form clear nanoemulsions with surfactants and co-surfactants sufficiently due to moderate lipophilicity and compatibility of the correct HLB and Smix ratio [12].

Table 4. Particle size, polydispersity index (PDI), and zeta potential of nanoemulsion formulations at different extract ratios and smix:oil compositions.

Extract Ratio	Smix : Oil	Sample	Appearance	Globule size (nm)	PDI	Zeta Potential (mV)
1:4	1:9	F1	Clear	1462	0.99	-38.76
	2:8	F2	Clear	1773	0.96	-1.15
	3:7	F3	Milky	284.7	0.49	-33.41
	4:6	F4	Milky	285.8	0.62	-23.95
	5:5	F5	Milky	322.5	0.47	-24.95
	6:4	F6	Milky	267.3	0.48	-26.7
	7:3	F7	Clear	51.15	0.25	-16.59
	8:2	F8	Clear	148.2	0.62	-11.28
	9:1	F9	Clear	101.6	0.64	-22.52
4:1	1:9	F1	Clear	1094	0.81	-30.36
	2:8	F2	Milky	879.2	0.70	-35.31
	3:7	F3	Milky	547	0.56	-30.4
	4:6	F4	Milky	263.5	0.69	-25.68
	5:5	F5	Milky	789.7	0.63	-26.07
	6:4	F6	Milky	305.5	0.44	-31.13
	7:3	F7	Milky	2193	0.99	0.15
	8:2	F8	Clear	68.52	0.21	-20.94
	9:1	F9	Clear	165.2	0.33	-23.32

3.5 *In vitro drug release*

Table 5. In vitro drug release profile of selected nanoemulsion formulations at different extract ratios.

Extract Ratio	Smix : Oil	Sample	Release Percentage		
			Hour 0	Hour 10	Hour 20
1:4	7:3	F7	35 %	85 %	50 %
	8:2	F8	10 %	18 %	25 %
	9:1	F9	26 %	31%	29 %
4:1	8:2	F8	30 %	70 %	45 %
	9:1	F9	15 %	24 %	31 %

Based on the results in Table 5, the best drug release was obtained in the formulation with an extract ratio of 1:4 (F7), which showed a rapid release with about 35% released in the first hour, a significant increase in release at the 10th hour reaching 85%, and a decrease in release at the 20th hour to 50%. In the formulation with an extract ratio of 4:1 (F8), a rapid release occurred with about 30% released in the first hour, a considerable increase in release at the 10th hour reaching 70%, and a decrease in release at the 20th hour to 45%. The release profile indicates a burst release, followed by maximum release and a subsequent decrease. This can be triggered by the degradation of active compounds or re-adsorption into the lipid phase [13].

In the drug release results, the formulation with an extract ratio of 1:4 (F8) showed much slower release, with about 10% released in the first hour, an increase of only 18% by the 10th hour, and 25% release by the 20th hour. The formulation with an extract ratio of 1:4 (F9) also showed a much slower release, with about 26% released in the first hour, an increase of only 31% by the 10th hour, and 29% release by the 20th hour. The formulation with an extract ratio of 4:1 (F9) exhibited a much slower release, with about 15% released in the first hour, an increase of only 24% by the 10th hour, and 31% release by the 20th hour. This release occurs due to a retarded condition caused by the more lipophilic nature of the *Persea*

americana Mill extract, which strongly partitions into the lipid core and delays the diffusion of active compounds into the release medium [14].

4 Conclusion

The development of a nanoemulsion-based formulation combining the traditional medicinal plants *Orthosiphon aristatus* (Blume) Miq. and *Persea americana* Mill. was successfully achieved, and demonstrated that the nanoemulsion formulation can be used as an agent to enhance the preventive anti-urolithiasis activity. Extract ratios of 1:4 (F7) and 4:1 (F8) yielded the best nanoemulsion formulations, based on formulation process and active compound release, which were the fastest and had the highest release. The findings of this study indicate that the nanoemulsion formulation can effectively enhance the solubility of active compounds, increase bioavailability, protect active ingredients from enzymatic degradation, improve the absorption of active substances, and strengthen its effectiveness as an anti-urolithiasis agent from *Orthosiphon aristatus* (Blume) Miq. and *Persea americana* Mill.

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