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IN VITRO ASSESSMENT OF ANTIUROLITHIATIC ACTIVITY OF APIGENIN WITH NITRIC ALGINATE VARIATION IN NANOSUSPENSION

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ABSTRACT

Kidney stones are hard kidney material, such as stones that originate from minerals and salts. Alternative therapy using medicinal plants as anticalculi is an option for treating kidney stones. One plant with anticalculi activity is celery, which contains various active chemical compounds, including apigenin. Apigenin is a diuretic that can help the kidneys remove excess fluid and salt from the body. However, apigenin has low solubility in water. To overcome the limited solubility of apigenin, nanosuspensions were prepared. This study aimed to formulate a nanosuspension, evaluate its preparation, and test it in vitro. This formula uses the concentration of the suspending agent, Na. The different alginates were F0 without active substances (0%) and F1 (1%), F2 (2%), and F3 (3%). The evaluation of the preparation includes tests for pH, viscosity, specific gravity, particle size, zeta potential, and adsorption efficiency. The results showed that the preparation had an average pH value of 4-7, an average viscosity value of 78-162 cP, a specific gravity of 0.98-1.02 g/mL, the preparation had a particle size of 928–974 nm, zeta potential of-26, 3 to -41.7, and an average entrapment efficiency of 99.63%-99.64%. Apigenin nanosuspension preparations can dissolve kidney stone calcium at 1800-7800 ppm. The test results showed that the apigenin nanosuspension has the potential to dissolve calcium kidney stones.

Keywords: kidney stones, apigenin, in vitro, nanosuspension

INTRODUCTION

Kidney stones are characterized by the formation of crystal stones in the pelvis or calyx. It is the most common cause of urinary tract abnormalities. The formation of kidney stones consists of both intrinsic and extrinsic factors. Intrinsic factors include age, gender, and heredity, whereas extrinsic factors include geographical conditions, climate, eating habits, substances contained in urine, and activity. To achieve rapid results, contemporary Western therapies, such as extracorporeal shock wave lithotripsy (ESWL), are indispensable. However, this often leads to undesirable consequences, such as kidney trauma, acute kidney injury, and hypertension. Although chemical drugs can relieve symptoms, they cannot completely eliminate the root of the problem, thus increasing the possibility of disease recurrence. Therefore, alternative approaches are essential for treating kidney stones (Rusdiana *et al*., 2019).

Extensive research has revealed the extraordinary potential of celery in protecting against a variety of health problems, including heart disease, liver disease, kidney problems, gout, and rheumatism. In particular, celery contains specific compounds that can effectively treat kidney problems, particularly by dissolving kidney stones (Rusdiana, 2018). Among various vegetable plants, celery contains apigenin, a non-toxic flavonoid. This compound has several beneficial properties including vasorelaxation, antiplatelet activity, and antioxidant effects. Apigenin has the potential to reduce the risk of coronary heart disease and improve endothelial function by lowering blood pressure and inhibiting platelet aggregation (Pusparini, 2015).

Nanotechnology, especially nanosuspensions, is an approach to overcome the limited solubility of apigenin. Nanosuspension refers to the dispersion of drug particles in colloidal form, with surfactants serving as stabilizers. Another definition of nanosuspensions is a two-phase system in which drug particles are dispersed in a carrier fluid, with suspended particles having a diameter of less than 1μm (Fadlilah and Gozali, 2018). Nanosuspensions offer several benefits, including increased solubility, dissolution rate, and bioavailability of drugs, as well as reduced dosage with minimal side effects, thereby improving patient compliance (Dzakwan and Priyanto, 2022).

Treatment of kidney stones can use thiazide diuretics and alkali citrate; however, scientific evidence for the success of therapy is not appropriate. In addition, kidney stone therapy can be performed with medical procedures for treating the disease, namely, surgery, wave technology applications (ESWL), and laser shooting, but each patient has different results and the costs are so high that only a small portion of society can afford it (Hu Chin Ong, 2014).Treatment efforts from nature with minimal side effects are needed, one of which is apigenin, a flavonoid that can break down and prevent attachment of calcium salt crystals or magnesium, which can cause kidney stone formation (Wientarsih, 2012).

RESEARCH METHOD

Tools and Materials

The tools used in this research were glass equipment, analytical scales, ultrasonic homogenizer, centrifuge, Vortex, PH meter AMT 20, Viscometer Brookfield, PSA Beckman Coulter, LS 13 320 type, and an atomic absorption spectrometer.

The materials used in this study included apigenin powder from Hefei Dielegance Biotechnology Co., Ltd, China, Natrii Alginate pa, 96% kidney stone calcium powder from a patient in Serang Banten City Regional General Hospital, 96% Ethanol, Aquadest, Aqua Demineral, HNO_3, H_2SO_4, and Batugin Elixir.

Procedures

a. Kidney Stone Content Test

Fine kidney stone powder (1 mg) was added to dilute the H_2SO_4 to form a white $CaSO_4$ precipitate. The precipitate was dissolved in hot, concentrated H_2SO_4, and then dissolved in ammonium sulfate.

b. Apigenin Nanosuspension Formulation

Table I. Apigenin Nanosuspension Formula (Stiani *et al.*, 2019)

Material	Concentration (%)					Function
	F0	F1	F2	F3	F4	
Apigenin	0	0,1	0,1	0,1	+	Active Ingridient
Etanol 96%	0	10	10	10	Batugin elixir	Solvent
Natrii alginat	1	1	2	3		Suspending agent
Aquadest	Ad 100	Ad 100	Ad 100	Ad 100		Solvent

Description:
F0 : Formula 0 (Natrii alginate 1%) ; F2 : Formula 2 (Natrii alginate 2%)
F1 : Formula 1 (Natrii alginate 1%) ; F3 : Formula 3 (Natrii alginate 3%)

c. Making Apigenin Nanosuspension Formula

Nanosuspension preparations were prepared using 100 ml of each formula. The ingredients were weighed according to their respective formulas. Apigenin was dissolved in 10 ml of 96% ethanol, stirred until homogeneous, 50 ml of distilled water was added, and the mixture was stirred again using a magnetic stirrer for 24 hours (solution A). Next, sodium alginate was mixed with distilled water until it expanded (solution B). Solution B was then mixed with solution A and stirred again using a magnetic stirrer for 24 hours. The procedure was repeated for all other formulas with different amounts of thickeners. Subsequently, the nanosuspension preparation was evaluated.

d. Evaluation of Apigenin Nanosuspension

1. Test pH

The pH of the preparations was measured using a pH meter. The pH meter electrode was dipped into the apigenin nanosuspension and left to stabilize. The pH values that appeared in the layer were recorded. Each formula must meet the requirements of the Indonesian Pharmacopoeia: 4-7 Nanosuspensi Apigenin (Fitriana *et al*. 2020).

2. Viscosity Test

A total of 100 ml of the apigenin nanosuspension was placed in a glass beaker. The viscosity was measured using a Brookfield viscometer with spindle number 3 at a speed of 30 rpm. According to SNI, the viscosity of nanosuspension is 37-396 mPa.s (Kartikasari *et al*. 2021).

3. Specific Gravity Test

The pycnometer was used to calculate the weight of the pycnometer containing the apigenin nanosuspension minus the weight of the empty pycnometer, which was then divided by the weight of the pycnometer containing distilled water minus the weight of the empty pycnometer. The specific gravity for preparations with water as a carrier must be > 1.00 g/mL, because water has a specific gravity of 1.00 g/mL (Wijaya and Lina 2021).

4. Determination of Entrapment Efficiency

Prepare 4 ml of nanosuspension at 1000 rpm for 4 minutes, and the residue is separated from the supernatant. The supernatant was homogenized with a vortex for 1 minute, and its absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 267.4 nm. The uptake was used to calculate the concentration of unadsorbed apigenin using a linear regression equation from the standard apigenin calibration curve. Replication was performed three times. Entrapment efficiency was calculated using the following equation:

$$\%\ \text{entrapment efficiency} = \left\{\frac{Ct - Cb}{Ct}\right\}$$

Description :
Ct = Total apigenin concentration in the nanosuspension
Cb = Concentration of unadsorbed apigenin

e. In Vitro Kidney Stone Decay Test

Kidney stones (100 mg) were soaked in 5 tubes containing 10 ml of Batugin elixir (positive control), apigenin nanosuspension with natrii alginate 0%, 1%, 2%, and 3% soaking was carried out for 5 hours at 37 °C with 15 minutes of shaking using a vortex, and then settled after incubation ended. A total of 7 ml of the filtrate from the soaking was digested with concentrated H_2SO_4: HNO_3 (v/v 2:1), shaken until homogeneous, and the volume was made up to 50 ml with demineralized water. In vitro measurements were performed using an atomic absorption spectrophotometer. The nanosuspension will be analyzed using a particle size analyzer.

RESULTS AND DISCUSSION

a. Kidney Stone Content

The results of examining calcium oxalate levels in kidney stones obtained in this study are shown in **Table II** certificate number 2405170020.

Table II. Kidney Stone Content

Inspection Name	Result
Number of stone	>10 Stones
Stone size	< 0,5-0,7 cm
Stone description	The stone is brown, has a rough surface and is irregular in shape
Composition	Calcium oxalate monohydrate (Whewellite) 79% Calcium oxalate dihydrate (Weddellite) 10% Calcium oxalate monohydrate (Dahllite) 10% Matrix (Unknow matter, usually protein) 1%
Stone Figure	

The results of the data analysis showed that 79 percentage of the kidney stones contained calcium oxalate. This is comparable to the results of research conducted by Dr. Nico A. Lumerta, K Nefro from Mediros Hospital, which was approximately 75-80%. So it can be said that the kidney stones used were positive for containing relatively high levels of calcium oxalate (Handayani, 2020).

b. Physical Evaluation of Apigenin Nanosuspension

1. pH Test

Table III. pH Test

Formula	pH			
	1	2	3	Average± SD
F0	6,77	6,71	6,65	6,71± 0,06
F1	7,01	7,01	7,00	7,00± 0,005
F2	6,66	6,71	6,67	6,68± 0,04
F3	6,74	6,83	6,81	6,79± 0,02

This shows that the pH of the apigenin nanosuspension preparation meets the criteria of the Indonesian Pharmacopoeia IV edition, namely 4-7. The pH value of the apigenin nanosuspension preparation is important because it greatly influences the performance of the nanosuspension preparation in the body. The pH determines the fraction of the drug that is not ionized so that it can be absorbed properly through a passive diffusion mechanism (Fitriana *et al*., 2020).

2. Viscosity Test

Table IV. Viscosity Test

Formula	Viscosity (cP)			
	1	**2**	**3**	**Average± SD**
F0	88,00	76,00	72,00	78,66± 6,3
F1	108,00	103,90	116,00	109,30± 6,1
F2	108,00	111,90	128,00	115,96± 10,6
F3	160,00	160,00	166,66	162,22± 3,8

Based on the results of tests on the formula with sodium alginate, the lowest viscosity was found in formula F0 (78.66 cP) and the highest viscosity was found in formula F3 (162.22 cP). The suspension viscosity value according to SNI is 37–396 cP; therefore, formulas 0, 1, 2, and 3 have a viscosity that meets SNI.Viscosity affects the stability of the preparation. A high viscosity helps reduce the sedimentation rate of particles by increasing the viscosity of the medium, thereby preventing particles from settling or clumping.

3. Spesific Gravity Test

Table V. Spesific Gravity Test

Formula	Spesific Gravity (g/mL)			
	1	**2**	**3**	**Average± SD**
F0	0,96	1,00	0,98	0,98±0,02
F1	0,97	0,99	1,00	0,98±0,01
F2	1,00	1,07	1,00	1,02±0,04
F3	0,98	0,99	1,02	0,99±0,02

Based on the table above, the specific gravity values for apigenin nanosuspension in formulas 0, 1, 2, and 3 with sodium alginate are respectively 0.98 g/ml, 0.986, 1.023, and 0.996 g/ ml (<1.00 g/ml) respectively, indicating that the preparation does not meet the specific gravity test requirements.

4. Particle Size

Table VI. Particle Size

Formula	Particle Size (nm)
	Sodium Alginat
F1	974
F2	953
F3	928

The particle sizes produced in nanosuspensions that used alginate in F1, F2, and F3 were 974, 953, and 928 nm, respectively. These results show that nanosuspensions that use sodium alginate have small particle sizes. The size of the nanoparticles determines how easily they can enter the cells. The smaller the particle size, the easier it will be to enter cells and the greater its absorption in the body (Mannuela *et al*., 2016)

5. Potential Zeta

Table VII. Potential Zeta

Formula	Zeta Potential (mV) ± SD
F1	-41.7 ± 0.3
F2	-29.3 ± 0.2
F3	-26.3 ± 0.2

Based on the results in **Table VII**, the zeta potential values produced by the three formulas were classified as good (± 30 mV and ± 20 mV). Particles that have a large zeta potential value, either positive or negative, will cause repulsive forces between particles, whereas, if the zeta potential value is low, it will cause attractive forces between particles, resulting in the particles joining together and becoming unstable (Akbari *et al*., 2011).

6. Efficiency Entrapment

Table VIII. Efficiency Entrapment

Formula	Efficiency Entrapment (%)
	Sodium Alginat
F1	99,64
F2	99,63
F3	99,64

The results showed that the nanosuspension had an expected adsorption efficiency of more than 50%. The results of this research were in the range of good adsorption efficiency, which is supported by the research by Arifin *et al*. (2022), where the adsorption efficiency results were more than 60%.

c. Calcium Decay in Kidney Stones In Vitro

Table IX. Calcium Decay in Kidney (Anticalculi)

Formula	Calcium Decay in Kidney (ppm)
F0	2300
F1	2400
F2	1800
F3	7800
K+	4400

Based on the results in **Table IX**, it can be observed that the smaller the particle size, the higher the calcium concentration. F0 was able to dissolve 2300 ppm of calcium, F1 2400 ppm, F2 1800 ppm, F3 7800 ppm, and F4 (positive control) at 4400 ppm. The highest calcium solubility was found in Formula 3 (7800 ppm). The viability of the apigenin nanosuspension was higher than that of the positive control. The test results showed that the apigenin nanosuspension had the potential to dissolve kidney stones.

Reducing the particle size increases the surface area. In accordance with Noyes-Whitney's law, the smaller the particle size, the greater the surface area so that the dissolution rate also increases. Apart from increasing the dissolution rate, reducing the particle size in the nanometer range can increase saturation solubility so that drug bioavailability can increase (Junyaprasert & Morakul, 2015).

The ability to dissolve calcium in kidney stones is caused by apigenin, which contains one of the compounds, flavonoids. The slavonoid compounds contained in apigenin are important factors in the solubility of kidney stone calcium. This is because the hydroxyl carboxylate group of flavonoid compounds reacts with kidney stone calcium to form a caflavonoid chelate complex that is easily soluble in water; thus, the water contained in urine helps eliminate kidney stones (Indriatmoko *et al*., 2022; Sinaga *et al*., 2019).

CONCLUSION

Based on the research that has been conducted, it can be concluded that apigenin can be formulated into a nanosuspension preparation that meets quality requirements. The value of the in vitro kidney stone decay test data on the entrapment efficiency of F1 (99.64%), F2 (99.63%), and F3 (99.64%) had an entrapment efficiency percentage of more than 50%. In Vitro test results showed that the formula with the highest potential for dissolving calcium was formula 3 (3%), with a Ca content of 7800 ppm compared to the positive control, which dissolved Ca at 4400 ppm. The viability of the apigenin nanosuspension was higher than that of the positive control. The test results showed that the apigenin nanosuspension had the potential to dissolve kidney stones.

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