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In vitro study of the effect avocado leaf and cats whiskers extractcombination as inhibitor of urine crystallization

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INTRODUCTIONUrolithiasis is presence of one or more calculi in urinary system. It is a disease with high recurrence both in human and small animal. Calcium oxalate is a major component of urolithiasis. The aim of this study is to evaluate the activity of combination extract *Persea americana* Mill and *Orthosiphon aristatus* (Blume)Miq. as inhibitor crystal calsium oxalate in urine through dissolving mechanism. **METHODS**The varian combination was compared with commercial brand (group K+) using artificial urine as solvent. This experiment used 11 group with varian combination *Persea americana*Mill. and *Orthosiphon aristatus* (Blume)Miqextract. The measurement of calcium from the solution was used spectrophotometric atomic absorption (AAS).**RESULTS AND DISCUSSION**The result showed that combination of extract *Persea americana* Mill. and *Orthosiphon aristatus* was effectively as inhibitor calcium oxalate and the combination of the extract at rasio (1:4) (P8) and (4:1) (P9) had the best potency as the inhibitor urolithiasis.
Keywords: *Persea americana* Mill, *Orthosiphon aristatus*(Blume)Miq., kidney stone, calcium oxalate,urolithiasis.

I. INTRODUCTION

Urolithiasis or kidney stones cases has taken the third rank in urinary system disorders after urinary tract infections and prostate abnormalities. This urinary system disorder affects nearly 12% of the world's population with 70-80% recurrence in men and 47-60% in women. As much as 80% of the urolith types found in humans are calcium oxalate types, both monohydrate and dihydrate (Sharma *et al*. 2016). Case studies in the veterinary world, especially in small dogs and cats, provides data information on the total number of calcium oxalate urolith cases in dogs and cats as much as 43% and 70.4%. The recurrence rate of calcium oxalate urolithiasis in dogs is quite high, which is 48-57% in 3 years. In cats, the recurrence rate is lower which is 6.8% in 2 years(Brown, 2017).

Calcium oxalate urolithiasis in humans, dogs and cats occurs when urine is in a supersaturated state with the presence of urine crystals. Generally calcium oxalate found in the kidneys can be in the form of calcium monohydrate (COM), calcium oxalate trihydrate (COT) and calcium dihydrate (COD). COM, has a more stable character and is more commonly found in the urinary system stones (Saha *et al.* 2013).The nucleation process takes place and under certain conditions attaches to other nucleus which will form a larger size crystal. This crystal will then grow and undergo aggregation with other crystals in a supersaturated solution. The condition of kidney injury and supersaturation is a major factor in the occurrence of kidney stones.

Kidney stone cases in the modern medical world today are treated through surgery, extracorporeal shock wave lithotripsy (ESWL), high power laser and the use of drugs such as diuretics and acidifyer/alkalinizer agents. This modern medical procedure is not affordable for all parties because of the limited facilities and uneconomical price (Sharma *et al.* 2016). Treating cases of kidney stones with these methods can cause serious complications such as acute renal injury and can increase the risk of shorter recurrence (Tiwari *et al.* 2012). Currently there are no drugs that can eliminate kidney stones andcan avoid repetition which makes preventive efforts become a preferred alternative by using herbs.

Avocado plants (*Persea americana* Mill) and cat whiskers (*Orthosiphon aristatus* (Blume) Miq) are plants commonly used by the community as traditional medicines for treating hypertension and urinary system problems. Several studies have been conducted in examining the activity of avocado leaves as diuretics (Adha *et al*. 2008), antilithiasis (Wientarsih *et al*. 2012), and abilityof increasing glomerular filtration rate in animals exposed to nephrotoxic substances (Madyastuti *et al.* 2015). Several studies that have been carried out on the

efficacy of cat whiskers (Orthosiphon aristatus) are antioxidants (Abdelwahab *et al*. 2011), anti-inflammatory (Yam *et al*. 2010), and diureticum (Hidayah*et al.* 2018).

The results of previous studies are used as a base for this study to evaluate the combination of avocado leaf extract and cat whiskers in dissolving calcium oxalate by *in vitro*. Optimization of the best combination ratio in providing calcium oxalate dissolving so that it can provide information on the effectiveness of the combination of the two extracts in preventing kidney urolith.

II. MATERIALS AND METHODS

Avocado leaf powder and cat whiskers plant, ingredients for phytochemical tests, the commercial trademark of antilitiasis Batugin Elixir®, calcium oxalate powder, and artificial urine ingredients.

Artificial urine production was made based on Fasano and Khan (2001) which was modified. The artificial urin composition are calcium chloride, $NaH_2PO_4.H_2O$, magnesium sulfate, magnesium chloride, sodium chloride, sodium citrate, and potassium hydroxide. Then each was weighed as much as 0.7771 gr calcium chloride, 5.7958 gr NaH2PO4.H2O, 0.0039 gr magnesium sulfate, 0.4781 gr magnesium chloride, 8.8218 gr sodium chloride, 0.5166 g sodium citrate, and 0.0099 gr potassium hydroxide. Dissolve each ingredient then place in a 1 L measuring flask and add distilled water to the boundary mark.

The research will be conducted in 11 groups, which are; negative control (K-), positive control (K +), avocado leaf extract 6000 ppm (P1), cat whiskers extract 5000 ppm (P2), and a combination of avocado leaf extract and cat's whiskers 1: 1 (P3), 1: 3 (P4), 3: 1 (P5), 2: 3 (P6) 3: 2 (P7), 1: 4 (P8), 4: 1 (P9). Commercial preparation of Batugin elixir ® used as positive control.2 mL of CaC_2O_4 solution is added in each test material that has been made and prepared. Add 19.8 mL of artificial urine so that the concentration is 100 times more dilute than the previous concentration. The solution is then measured using an *absorbance atomic spectrophotometer* (AAS) at a wavelength of 422.7 nm. The remaining mixture of the test material with CaC_2O_4 solution in a closed test tube was allowed to stand for 3 hours and was shaken every 15 minutes.

Data results from the measurement of calcium oxalate levels with AAS were then statistically tested using ANOVA test at 5% level with SPSS 17.0 portable program. Further testing was carried out with Duncan's test if the results of ANOVA analysis showed results that were significantly different between treatment group and control group.

III. RESULTS AND DISCUSSION

Qualitative Analysis of Phytochemical Compounds

Table 1 shows the phytochemical screening results of avocado leaf extract and cat's whiskers. Based on the results of phytochemical test qualitatively, avocado leaf extract contains flavonoids, phenolics, alkaloids, saponins, and tannins, while cat's whiskers contain flavonoids, phenolics, saponins, and tannins. This positive result is based on the formation of deposits or changes in color that occur.

Table1 Phytochemical test results of avocado leaf extract and cat's whiskers.

Phytochemical test	Exract	
	Avocado Leaf	Cat's whiskers
Alkaloid	-	-
Flavonoid	+++	+++
Phenolic	++++	+++
Saponin	+++	++++
Tanin	++++	++++
Steroid	-	-
Triterpenoid	+	-

The results of qualitative examination of phytochemical compounds in both extracts are in accordance with the examination carried out by Yasir et al. 2010 and Adnyana et al. 2013.

Quantitative analysis of flavonoids and phenolic

Table 2 below shows the quantitative analysis of flavonoids and phenolics. The procedures below are in accordance with the procedures of the Indonesian Ministry of Health, (2000).

Sampel	Amount of flavonoid/100 g	Amount of fenolik/100 g
Avocado leaf extract	5.00	4.07
Cat's Wiskers extract	6.00	6.72

Based on certain studies, the content of secondary metabolites of flavonoids and phenolics play a role in the process of inhibiting crystallization of calcium oxalate (Suharjo and Cahyono 2009, Gurocak *et al*. 2006, Chauhan R *et al.* 2013). The presence of phenolic in avocado leaves is in accordance with research conducted by Carpena *et al.* 2011, which tested phenolic from fractionation of polar and non-polar solvents as antioxidants and antilipids.

Analysis of Calcium Oxalate Solubility

Measuring calcium ions from calcium oxalate decay was carried out three times (triplo) each at the 0 and 3rd hours. The results of calcium measurements using AAS are presented in Figure 1.

Table 3 Calcium Measurement in all treatment groups

Groups	Average amount of Ca (ppm)		Amount of dissolve Ca (ppm)
	0 Hour	3rd Hour	
K (-)	751.15±3.23^{b}	877.01±10.87^{c}	125.86
K (+)	831.59±8.34^{cd}	1063.26±10.90^{g}	231.67
P1	843.26±4.65^{cd}	1031.25±5.18^{e}	187.99
P2	846.45±16.26^{d}	1086.59±5.23^{h}	240.14
P3	902.91±5.09^{e}	784.86±19.02^{a}	-118.05
P4	930.48±16.01^{f}	842.98±8.54^{b}	-87.50
P5	967.43±11.49^{g}	849.02±10.52^{b}	-118.41
P6	971.31±20.10^{g}	898.12±10.31^{d}	-73.19
P7	968.05±4.22^{g}	911.52±13.33^{d}	-56.53
P8	720.48±18.46^{a}	1040.90±10.23^{f}	320.42
P9	821.87±11.11^{c}	1015.62±11.71^{e}	193.75

Information:

K(-)=Negativecontrol

K(+)=Positivecontrol

P1=Groupofavocadoleaf (DA)extractdoseof 6000

P2=Groupofcat'swhiskers(KK) extractdose of5000

P3 = DA:KK(1:1)=CombinationgroupofDA extract6000ppmand KK 5000ppm

P4 =DA:KK(1:3)=CombinationgroupofDA extract6000ppmand KK 15000ppm

P5 = DA:KK(3:1)=CombinationgroupofDA extract18000ppm and KK 5000 ppm

P6 =DA:KK(2:3)=CombinationgroupofDA extract 12000ppmand KK15000ppm

P7= DA:KK(3:2)=CombinationgroupofDA extract18000ppm and KK10000ppm

P8 =DA:KK(1:4)=CombinationgroupofDA extract 6000ppm andKK20000ppm

P9 = DA:KK(4:1)=CombinationgroupofDA extract 24000ppmandKK5000ppm

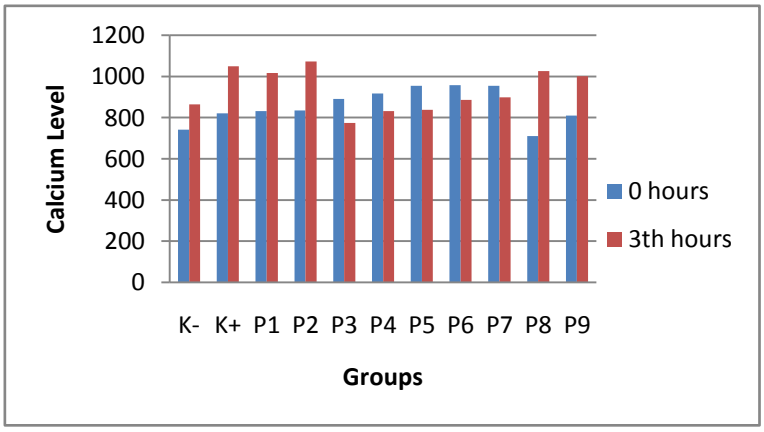

Figure 1 Calcium Content all treatment groups

Calcium oxalate in urine can take form of calcium oxalate monohydrate (COM), calcium oxalate dihydrate (COD) or calcium oxalate trihydrate (COT). COT is a crystal in an unstable form, in certain conditions it will turn to COM. Calcium oxalate used in this study is mostly crystalline in COM form. Process nucleation was occurred by spontaneously for COM in hipercalciuria situation. In the treatment group P3, P4, P5, P6, and P7 the calcium level in the 3rd hour was lower than in the 0 hour, there was not dissolution activity. It was suspected that the nucleation process is still occuring to obtain a stable formation. Nucleation occurs due to a decrease in zeta potential in urine artificial solution (AU) (Beghalia *et al*. 2007). Some studies suggest that extracts generally work in inhibiting crystallization by encouraging changes in COM to COD (Fouda *et al*. 2006 and Saha et al. 2013).The form of calcium oxalate crystals is presented in Figure 2.

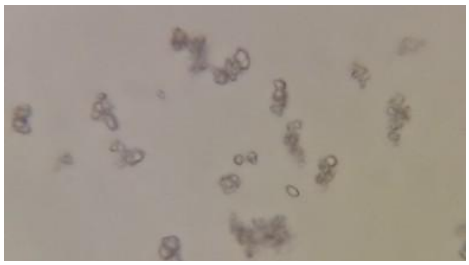

Figure2 Majority of forms crystal calcium oxalate

Based on the measurement of dissolved calcium using the AAS instrument, the highest yield was shown by the P8 group followed by K+ and P9. Calcium dissolves at P8, K

+ and P9 through the formation of bonds with flavonoids. The more the flavonoid levels, the greater the decay of calcium oxalate. This is because the OH group found in flavonoid compounds is able to react and bind to calcium in crystals and form complex compounds of Ca-flavonoid compounds, so that these compounds will dissolve more easily in water (Suharjo and Cahyono 2009). The dissolved calcium level in P8 is 320.48 ppm while that in K + is lower at 231.67 ppm. Statistically the 3-hour calcium level at P8 and P9 against K + was statistically significantly different ($P < 0.05$).

According to Gurocak *et al.* 2006, saponin and phenolic compounds contained in the extract were able to act as anti crystallization. Saponins are known as inhibitor process of calcium oxalate crystal by disaggregating mucoprotein that promote crytsallisation. A single extract of cat's whiskers has a greater decay effect compared to avocado leaf extract because it has a higher total flavonoid and saponin content than avocado leaf extract. The highest dose combination also provides dissolution of calcium oxalate crystals which are characterized by high levels of calcium which are almost equivalent to commercial preparations. Statistically equivalent, compared with negative groups were significantly different ($P < 0.05$).

MICROSCOPIC OBSERVATION

Observation of crystal images in the treatment group presented in Figure 3 below is carried out descriptively. In K- as a negative control, crystals are quite dense because they do not get the treatment. The density of calcium oxalate crystals in the group given extracts and K + is reduced due to the inhibition of nucleation and aggrigation processes. The dissolution of calcium oxalate crystals at P8 and P9 is in line with the increase in dosage.

Based on microscopic observations the use of a combination of avocado leaf extract and cat's whiskers at a ratio of 1: 4 and 4: 1 is able to dissolve calcium oxalate crystals so as to reduce the number of crystals and prevent the aggregation process. This is in accordance with the research of Zhong et al. 2012, which states that total flavonoids and phenolics can inhibit crystal aggregation and reduce crystal size.

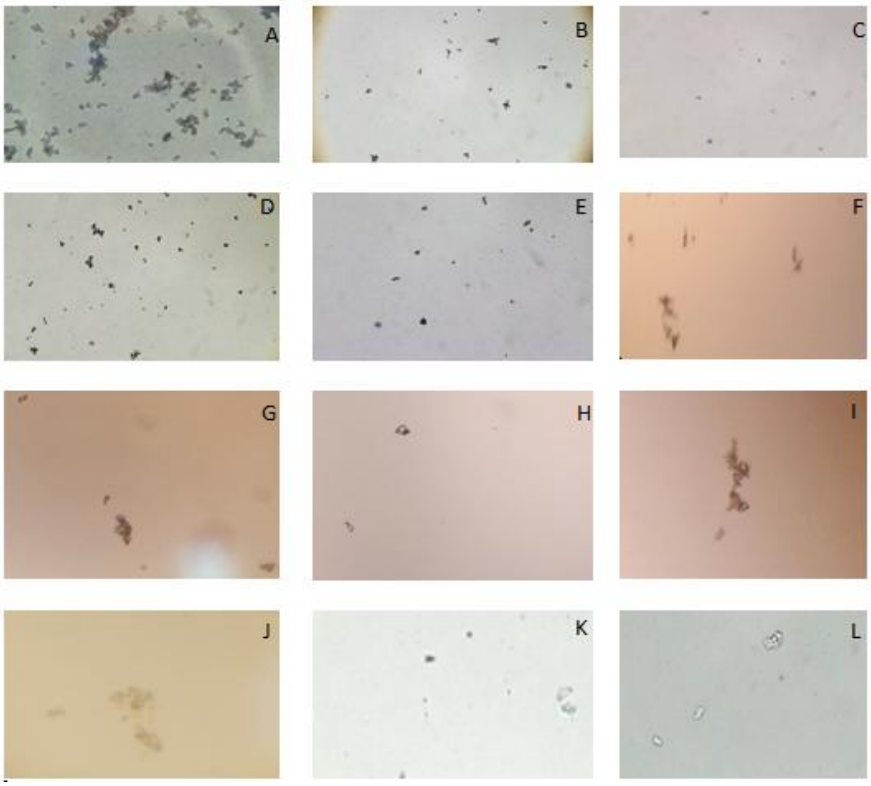

Figure3Observationofcalciumoxalatecrystalsinthe3rdhour

(A)Beforetreatment, (B) groupK-,(C)groupK+,(D) group P1
(E)group P2, (F)group P3, (G) group P4, (H) group P5, (I) group P6,
(J) group P7, (K) group P8, (L) group P9.
Undermagnification400xmicroscope.

IV. CONCLUSIONS AND SUGGESTIONS

Conclusion

The combination of extract of Avocado leafs and cat's whiskers are able to dissolve calcium oxalate crystal by in vitro. Flavonoid and phenolic compounds in both extracts plays a role in inhibiting crystallization by preventing the calcium oxalate crystal aggregation process

Suggestion

Further research is needed using the same concentration levels between extracts of calcium oxalate decay. The artificial urine used should always be in a fresh state and according to the standard the reseach need calcium oxalate crystalline powder or use the kidney stones directly.

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