Molecular Docking Simulation and in vivo Assay on Anti-Nephrolitic Potency of Avocado Leaf and Cat’s Whiskers Extract

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Abstract

The aim of this study was to evaluate the activity of Avocado (Persea americana Mill) leaf extract and Cat whiskers (Orthosiphon aristatus (Blume)Miq) extract combination as calcium oxalate nephrolithiasis inhibitor through in vivo assay and molecular docking simulation. Commercial brand Batugin Elixir® (BG) was used as positive control group. Thirty five rats were divided into 7 groups i.e normal (n=5), EG as inducer group (n=5), BG as positive control BG (n=5), avocado leaf extract with 300 mg/kgBW dose (AV) (n=5), whisker cats extract with 250 mg/kgBW dose(OR) (n=5), combination dose 1 containing AV 300 mg/kgBW and OR 250 mg/kgBW (Mix1) (n=5) and combination dose 2 containing AV 600 mg/kgBW and OR 500 mg/kgBW (Mix2) (n=5). Biochemical variables measured in rat serum were urea and creatinin. Urine Ca, Mg, pH and urine volume were also measured. Histopathology of kidney was analysed in order to evaluate protective effects of the treatment and crystal dissemination in the tissue. The result showed that group of Mix1 showed good activity performance by lowering urine calcium level (P<0.05) and increasing urine magnesium level (P<0.05), increasing urine volume, and modify urine pH. Increase of urea and creatinin from group Mix1 are suppressed compared to normal group. The other group result showed lower activity than group Mix1. Molecular docking simulation showed that active compounds such as quercetine and rosmanic acid occupied the active site of receptor glycolate oxidase (GO). The conclusion of this study was grup Mix1 had the best activity as inhibitor of nephrolithiasis.

Key words: Molecular docking simulation, antinephrolitic, in vivo, Persea americana Mill, Orthosiphon aristatus

INTRODUCTION

Nephrolithiasis or kidney stone is one of the more often multifactorial kidney diseases which globally happen to 10% of world population with increasing trend every year (Tsujihata 2018). Recurrence rate of this disease is very high, being 70-81% in male and 47-60% in female after first kidney stone elimination (Thakur et al. 2013). Recurrence of nephrolithiasis in both human and animal is the main problem of the disease and currently no satisfying solution is available. Kidney stone treatment with repeated invasive procedure may cause serious complications such as acute renal injury and increased recurrence risk by 10% (Liang et al. 2016). Nephrolithiasis recurrence prevention with herbal medicine is hoped to be an alternative treatment for nephrolithiasis case. According to information passed down for generations, people have been using herbs such as cat whisker, field milk thistle, and gripeweed to treat kidney stone problem (Pardede et al. 2018).

Avocado plant (Persea americana Mill) and Cats whiskers (Orthosiphon aristatus) are plants often used by communities as traditional drug in treating hypertension and urinary tract disorder. Several activity tests have provided information that avocado leaf is beneficial as diuretics (Prasetyo et al. 2010), anti-lithiasis (WIentarsih et al. 2012), nephroprotective (WIentarsih et al. 2015) and increases glomerulus filtration rate (Madyastuti et al. 2015). Secondary metabolite compound as avocado leaf marker is quercetin. Quercetin has the capacity as antioxidant better than rutin, isohamertin, and apigenin (Owolabi et al. 2010) and also reported to have anti-inflammatory activity (Adeyeni et al. 2002). The potency of cats whiskers also noted as diuretics, anti-inflammatory, and lowering blood pressure (Prayoga 2008), anti-lithiasis (Zhong et al. 2012), anti-oxidant, and anti-apoptosis (Abdelwahab et al. 2011). The main markers of cat whiskers are sinensetin and rosminic acid (Almatar et al. 2014).

Although each plant has its own active substances, until now the activity of avocado leaf and Cat whiskers extract combination in inhibiting nephrolithiasis as prevention to recurring nephrolithiasis has not been reported. Docking simulation report to predict the mechanism of the two extract in inhibiting calcium oxalate crystal is also unavailable. This research’s objective is to evaluate the effective dosage as anti-nephrolithiasis of avocado leaf and cat whiskers extract combination on animal model with induced nephrolithiasis with molecular docking simulation to predict the mechanism of extract activity.
MATERIALS AND METHOD

**Materials**

Tools used are High Performance Liquid Chromatography (HPLC), spectrophotometer AAS, light microscope, polarization microscope Meiji MT6300H Fluorescence Trinocular and Abaxis® VetsScan Veterinary Blood Chemistry Analyzer.

Materials used are avocado leaf, cat whiskers plant, commercial prepatre of Batugin Elixir® (PT Kimia Farma), white male Sprague Dawley, solvent ethanol 70%, aquades, propylene glycol, Emplura® (ethylene glycol, Merck CAS No :07-21-1), EMSURE® (ammonium chloride, Merck Index no : 017-014-0008), ketamine, xylazine, natrium oxalate, calcium chloride, natrium chloride, natrium acetate, concentrated HCI, primary antibody osteopontin (Rabbit polyclonal to OPN ab8448 Abcam Cambridge UK), and Secondary antibody (SP link HRP Broad DAB Bulk Kit Lot.No.K186818A).

**Phytochemical extraction and filtering**

Simplicia of avocado leaf and cat whiskers in powder form was acquired from Herb and Medicinal Plant Research Center (Balai Penelitian Tanaman Rempah dan Obat—BALITRO) Bogor, Indonesia. Plant determination was conducted in Herbarium Bogoriense LIPI Bogor, Indonesia. Extraction method used was maceration with solvent ethanol 70% for 3x24 hours. Sample and solvent comparison was 1:1. The extract obtained was thickened by rotary evaporator in 40C temperature and 50 rpm speed until concentrated extract was obtained, before phytochemical filtering followed through Harborne method (1987).

**Quercetin and rosminic acid docking simulation**

Quercetin and rosminic acid docking simulation Procedure began with protein structure preparation, consisting glyolate oxidase receptor and the tested ligands quercetin and rosminic acid. The three dimensional (3D) structures of glyolate oxalate and comparison ligand were obtained from Protein Data Bank (PDB) in http://www.rscb.org, (ID code= 2RDT).

The structure of tested ligands quercetin Pubchem CID 5280343 and rosminic acid Pubchem CID 5281792 were obtained from http://pubchem.ncbi.nlm.nih.gov. Ligand structures were transformed into 3D structure by software Marvin View 6.0 and stored in PDB format. All ligand used were optimized by software AutoDock Tools1.5.6 through the addition of hydrogen atom and stored in PDBQT format. Receptor used was identified by software Discovery Studio Visualizer v. 16.1.0.15350. Molecule interaction analysis consist of hydrogen bonding analysis and two dimensional (2D) hydrophobic interaction was conducted by software Ligplot +4.3.5. Analysis was done by comparing energy affinity while the visualization of ligand attachment site on receptor was compared its comparison ligand.

**Animal and experimental protocols**

The study was approved by the Animal Ethic Commission of Faculty of Veterinary Medicine with Number : 095/KEH/SKE/V III/2018 on August 31th 2018. Adult male Sprague Dawley rats (200-250 g) obtained from BPOM (Badan Pengawasan Obat dan Makanan) were then maintained under standard laboratory conditions (Temperature: 25°C, Humidity 60% and Light regimen 12 h dark cycle). The animals had free access to water ad libitum and fed standard diet during 1 week acclimatization period. The rats were then divided into 7 groups, with each group consisted of 5 animals. The control group as normal treatment received drinking water. Whole animal were treated by inducer (0.75% (v/v) ethylene glycol and 2% (b/v) therewith the material test except control group. The EG group received inducer agent only. The BG group as positive control received inducer and treated with commercial brand Batugin Elixir®. The group EG+AV received inducer and treated with extract avocado leaf at a dose of 300 mg/kgBW and EG+WJ group received inducer and treated with extract whisker jaws extract at a dose of 250mg/kgBW. The remaining groups were group Mix1 which received inducer and combination extract at dose I (EG+AV dose 300mg/kgBW+WJ dose 250mg/kgBW) and group Mix2 which received inducer and treated dose II (EG+AV dose 600mg/kgBW+WJ dose 500mg/kgBW). All groups were given inducer ad libitum in drinking water and extracts concomitant given by oral once a day for 28 days.

**Urine collection and lithogenic factor measurement**

Urine was collected for 24 hours via the use of metabolite cage on day 13 and 26 of treatment period. Calcium concentration, magnesium concentration, pH, and urine volume were also measured. One-time urine collection was also conducted to observe microscopic urine crystal.

**Kidney function serum analysis**

Blood sample was obtained on week 0, 2, and 4 of treatment period from coxigealis vein by the tail. Rats were separated from their groups then fasted for 8-10 hours. Anesthetic was administered via intraperitoneal injection with ketamine and xylazine (75-100 mg/Kg : 5-10 mg/Kg). In the last day, blood was taken from intracardial route after priorly anesthetized and followed by euthanasia with cervical dislocation method. Blood samples were left to rest in room temperature for some time before centrifuged in 1000 rpm for 10 minutes. Urea and creatinine concentration in serum was done by Abaxis® VetsScan Veterinary Blood Chemistry Analyzer instrument.

**Kidney histopathology**

Kidney tissues were fixated in BNF 10% fixative liquid. Tissues were dehydrated in graduated alcohol (70%, 80%, 90%, and absolute alcohol) and followed with clearing in xylol I, II, and III and then embedding into paraffin. Resulting paraffin blocks were cut in ± 5µm thickness by microtome. The preparates were then deparaffinized and rehydrated for Hematoxylin Eosin (HE) staining. Kidney histology examination from HE staining was done by light microscope and polarization microscope.
Data Analysis
All quantitative data analysis were done by software SPSS 16.0 with one-way ANOVA variety analysis method and presented in average and standard deviation. The results were process with Duncan multiple range test (P<0.05) to see the difference between treatment group.

RESULT

Phytochemical filtering
The result of avocado leaf and cat whiskers phytochemical filtering is provided in Table 1. The two extracts were positive for containing flavonoid, phenolic, saponin, tannin and triterpenoid. Cat whiskers has higher saponin content qualitatively compared to avocado leaf extract.

Molecular docking simulation
Figure 1 showed the result of molecular docking simulation analysis between tested ligands quercetin (CID 5280343) and rosmarinic acid (CID 5281792) with glycolate oxidase receptor (GO) code 2RDT. The simulation result of molecular docking produced the affinity energy data for quercetin, rosmarinic acid, and inhibitor ligand CDST of -8.2, -7.9, and -6.9 kcal/mol respectively. The affinity energy data value showed that quercetin and rosmarinic acid ligand have stronger attachment compared to inhibitor CDST ligand. Based on the previous binding data, quercetin and rosmarinic acid can move natural inhibitor ligand 4-carboxy-5-dodecylsulfanyl-1,2,3-triazole (CDST) and may potentially be used as prepare in treating overproduction of oxalate. The active site of GO receptor consist of Tyr 208, Leu 205, Leu164, His260, Arg263, Arg167, Val209, Tyr26, Tyr132, Thr161, Trp110, and Lys176. As much as 84.61% of amino acid in binding sites has strong interaction with tested ligand rosmarinic acid and 77% has strong interaction with quercetin.

Urine lithogenic factor variable
The result of fresh urine lithogenic factor and accumulation for 24 hours is provided in Table 1. The urine lithogenic factor measurement showed calcium concentration in urine on almost all extract treatment group and commercial preparate group showed significant difference compared to EG group (p<0.05). EG+AV group showed higher Ca concentration compared to normal and EG group.

<table>
<thead>
<tr>
<th>Test Sample</th>
<th>Test Sample</th>
<th>Flavonoid</th>
<th>Phenolic</th>
<th>Saponin</th>
<th>Tannin</th>
<th>Triterpenoid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avocado leaf extract</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cat whiskers extract</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 1 The result of avocado leaf and cat whisker extract phytochemical filtering

<table>
<thead>
<tr>
<th>Group</th>
<th>Calcium (mg/dl)</th>
<th>Magnesium (mg/dl)</th>
<th>Urine volume (ml)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.45± 0.70d</td>
<td>10.87 ± 1.20e</td>
<td>8.22±1.77cd</td>
<td>7.0</td>
</tr>
<tr>
<td>EG</td>
<td>6.72 ± 2.65c</td>
<td>31.46 ± 4.35c</td>
<td>5.87±0.88e</td>
<td>5.5</td>
</tr>
<tr>
<td>BG</td>
<td>3.47 ± 1.39a</td>
<td>52.62 ± 2.08e</td>
<td>5.80±0.78e</td>
<td>8.0</td>
</tr>
<tr>
<td>EG+AV</td>
<td>10.28 ± 1.42d</td>
<td>13.38 ± 0.66c</td>
<td>7.15±0.49e</td>
<td>8.5</td>
</tr>
<tr>
<td>EG+WJ</td>
<td>4.40 ± 0.65c</td>
<td>32.51± 4.27f</td>
<td>6.80±0.42e</td>
<td>7.5</td>
</tr>
<tr>
<td>Mix dose 1</td>
<td>3.80 ± 0.89b</td>
<td>73.14 ± 0.56c</td>
<td>8.85±0.49d</td>
<td>8.0</td>
</tr>
<tr>
<td>Mix dose 2</td>
<td>6.42 ± 1.39c</td>
<td>24.52 ± 3.01d</td>
<td>6.75±0.35e</td>
<td>6.5</td>
</tr>
</tbody>
</table>

Annotation: Data is showed as average ± standard deviation. Different superscript on the same column indicated significant difference (P<0.05)
Mg concentration in urine on all treatment group have significant difference compared to control and EG group (P<0.05). Mix1 group showed significant increase (P<0.05) compared to other groups. Urine volume showed EG+AV and Mix1 groups are significantly different with EG (P<0.05). Volume for group EG, AV and Mix1 respectively are 5.87 ml, 8.85 ml and 7.15 ml. Volume of group BG, EG+WJ, and Mix2 are not significantly different with EG group (P>0.05). EG group pH measurement informed that rat pH urine is 5.5. Extract treatment was able to correct pH to relatively alkaline.

Fresh urine crystal observation in treatment group is presented in Figure 2. Based on descriptive observation in group EG (Figure A), there are many crystals found within urine. Crystal shape appears to be calcium oxalate dihydrate (COD) in the form of octahedral and dodecahedral within the urines belong to group EG, EG+AV, and Mix2. D group crystal appears to look similar to needles, which means they are crystals in COT form that are very labile. The urine crystal in EG+WJ and Mix1 are fewer and smaller compared to other group. Control group are normal group with no crystal throughout the period of this research.

Figure 2: Crystal urine in treatment group with inducer and tested sample extract. Magnification 20x. (A) EG Group, (B) BG group, (C) AV group, (D) OR group, (E) mix dose 1 group and (F) mix dose 2 group.

Figure 3: Creatinine and urea concentration (mg/dl) in every treatment group serum on day 14 and 28. Figure A and B: urea and creatinine serum for every treatment group. Figure C and D: Δ urea and creatinine in percentage (%)
Figure 4  Histology observation on control and treatment group, Haematoxylin eosin staining. Magnification 20x.
Annotation : A; Control group, B; EG group with calcium oxalate crystal by its lumen
(*) protein within the lumen (black arrow), C; BG group, D; EG+AV group, E; EG+WJ group, F; Mix1
 group, and G; Mix2 group

Figure 5 Crystal luminescence in kidney samples by using polarization microscope for each treatment group.
Figure A : (EG Group), B (BG Group), C (EG+AV Group), D (EG+WJ Group), E  (Mix1 Group) and F
(Mix2 Group). 20x Magnification.

BUN and creatinine serum value
The result of rat serum creatinine and urea measurement from every treatment group is provided in Figure 3. Rat
creatinine and urea concentration on every treatment group on day 28 showing increasing trend. Ureum and creatinine
concentration increases in EG group with its highest being 274.34% and 110%. Extract administration on every
treatment group able to press the increase of ureum value with significantly different value against EG group
(P<0.05). Ureum value measurement in day 28 is highest seen in BG group with commercial preparate and
statistically different with other groups (p<0.05). Creatinine value on treatment group can be pressed from
increasing but their values do not significantly differ with control and EG group (P>0.05). Mix1 group was able to
lower the increase of ureum value for 14 days into the treatment period as much as 23.25% and lower creatinine
value for 4.61%.

Rat kidney histopathology observation
Figure 4 showed kidney histopathology of rats from treatment group. In EG group (Figure B), crystals were found within the lumen of tubulus with many of tubules cells appears necrotic. Protein deposition, inflammatory cell invasion, and tubulus lumen dilatation are found compared to control group. Mix1 group was able to provide nephroprotective activity against nephrotoxic inducer with minimum lesion expression. Kidney histopathology image from commercial prepare BG showed hemorrhage by interstitial space on day 28. This means BG prepare is not advised to be consumed continuously for long period of time because of its irritant effect and the risk of hemorrhage.

Crystal luminescence in kidney observation
The result of crystal luminescence observation in treatment group by using polarization microscope is provided in Figure 5. EG (Figure A) showed extensive spread of crystal with larger quantity compared to other treatment, such as BG in figure B, EG+AV in figure C, EG+WJ in figure D, Mix1 in figure E and Mix2 in figure F. The number of crystal in Mix1 figure appears fewer and smaller compared to EG, BG, and Mix2 group.

DISCUSSION
The number of nephrolithiasis patients tends to increase along with the change of lifestyle, which often is the cause of increasing metabolic syndrome cases (Sakhaee, 2008). These days, communities tend to choose herbal option with anti-nephrolithiasis as recurrence prevention, along with a change of lifestyle. Several advantages of herbal medicine are its relatively affordable price, generally safer, and even if side effect appears it is usually minor side effects (Ahmed et al. 2013, Bashir et al. 2009). This research used the combination of avocado leaf and cat whiskers extract to lower the recurrence of nephrolithiasis in rats.

Phytochemical contained in avocado leaf extract and cat whiskers extract are flavonoid, saponin, tannin, triterpenoid, and phenolic (Table 1) which are reported to be able to prevent the formation of urine crystal (Gurocak dan Kupeli 2006). Derivates of flavonoid and phenolic contained in the leaf and seed of avocado are: queretin 3-O-glucoside, kaemferol, rutin, isoaharmnetin glycoside, catechin dan epicatechin (Melgar et al. 2018). Several flavonoid derivatives consist in cat whiskers are sinensetin, eupatirin, 3-hydroxy-5,6,7,4-tetrametoksiflavone, rosmarinic acid, and cichoric acid (Zong et al. 2012).

The predisposition of calcium oxalate nephrolithiasis is hyperoxaluria and hypercalciuria in supersaturated condition. Glycolate Oxidase (GO) enzyme is an enzyme found in liver that can change glycolate substrate into oxalate. When substrates are over abundant, oxalate will also be formed equally numerous, followed the formation of calcium oxalate crystal (Shirfule et al. 2011). One of the efforts to prevent hyperoxaluria is by inhibiting GO enzyme. Currently this enzyme can be inhibited if oxalate production did not occur, which cause calcium oxalate crystal to not nucleate in kidney. Molecular docking simulation result (Figure 1) showed that the two tested ligands, quercetin and rosminic acid, were able to induce amino acid on the active side of GO receptor and showed competitive antagonist activity. The two tested ligands showed good docking score compared to their natural inhibitor 4-carboxy-5-dodecylsulfanyl-1,2,3-triazole (CDST) so it is predicted that they can inhibit GO enzyme well. Avocado leaf and cat whiskers extract have the potency as anti-hyperoxaluria and thus may prevent calcium oxalate nephrolithiasis.

Calcium oxalate in kidney is a chain of complex processes begun by supersaturated condition in kidney which progress to nucleation, crystal growth, aggregation, and retention in tubule cells. Several risk factors of calcium oxalate nephrolith are hypercalcuiuria, hyperoxaluria, hypocitraturia, low magnesium, and suitable pH condition (Stoller and Meng 2007). Urine lithogenic factor measurement (Table 1), showed that low dose combination group (Mix1) can lower calcium level in the urine, increase urine magnesium lever, increase urine volume, and shift the pH level and thus able to suppress predisposition factors of calcium oxalate crystal formation. Calcium oxalate can be easily formed in around pH 4 and only few in pH 8 (Manissorn et al. 2017). COM crystal formation process has high possibility to be formed in pH 4 and is pathogenic for crystal and tubule cells affinity is high. The shift of pH into alkaline is among preventive effort to inhibit the formation of calcium oxalate nephrolith. Acidic urine is a very conducive condition for calcium oxalate crystal formation, specifically for its aggregation and crystal deposition within the urinary system (Huang et al. 2006).

Calcium oxalate crystal in urine may form monohydrate (COM), dihydrate (COD), and trihydrate (COT) (Yamaguchi et al. 2005). Hexagonal COM crystals are often found in induction group (EG) in this research. Administration of extract containing flavonoid may influence the solubility of calcium oxalate to be more soluble. The presence of OH group in flavonoid can form a chelating compound between calcium and flavonoid compound and thus able to inhibit aggregation process and calcium oxalate crystal cell retention (Li et al. 2015). This is supported by the result of this research as in combination treatment group, formed crystals are less dense, small, and not in hexagonal form which can easily flow away in urine (Figure 2).

Oxidative stress condition caused by hyperoxaluria and the presence of calcium oxalate nucleus in pathologic level caused renal tubular damage followed by tubular obstruction. This condition caused a drop of glomerulus filtration rate, which impact elimination of urea and creatinine metabolism product (Sridharan et al. 2016, Bayir et al. 2011, dan Divakar et al. 2010). This condition caused an increase on urea creatinine level in the blood, which was shown on negative control group of this research which tend to be higher than control (Figure 3). Administration of extract combination can inhibit the rising urea and creatinine level in treatment group and increase glomerulus filtration rate. The use of commercial prepare BG in long period of 28 days must be done.
carefully and kidney function examination is advised. Based on empirical data, the use of *Strobilanthi crispsus* for a long period of time and within certain dosage may cause irritation and hematuria. This is supported by our observation of kidney histopathology, where in the BG group interstitial hemorrhage was found in large number (Figure 4 C). Observational result of kidney histopathology (Figure 4) showed lesions by kidney tissue which are necrotic cells in tubules, the formation of protein deposition, hemorrhage, infiltration of inflammatory cells, and dilatation of tubules lumen (EG group). Crystal deposition within tubules lumen caused tubules obstruction. This is a compensation effect of being exposed to nephrotoxic material which caused acute tubular necrosis and turned into acute kidney injury. Administration of herbal medicine can prevent kidney lesions by anti-oxidant activity, anti-inflammatory activity, suppressing oxidative injury marker MDA, and tubule lumen dilatation. The mechanism of calcium oxalate nephrolithiasis inhibition by avocado leaf and cat whiskers extract was through the inhibition of glycolate oxidase enzyme which in turn preventing hyperoxaluria condition.

**CONCLUSION**

The result of this research showed that the combination of avocado leaf (250 mg/Kg BW) and cat whiskers (300 mg/Kg BW) extract (Mix1) was able to inhibit calcium oxalate crystal formation in rats with induced nephrolithiasis, through increasing glomerulus filtration rate and thus increasing urination volume, maintaining lithogenic factor balance in urine, and suppressing tubules epithelial cell by antioxidant and anti-inflammatory activity. Kidney tissue histopathology image also showed how Mix1 group has protective activity to kidney which minimize lesions such as necrotic cell, protein deposition, and tubule lumen dilatation. The mechanism of calcium oxalate nephrolithiasis inhibition by avocado leaf and cat whiskers extract was through the inhibition of glycolate oxidase enzyme which in turn preventing hyperoxaluria condition.

**Gratitude**

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