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Antioxidant activity of unpolished Riceberry rice (*Oryza sativa*) and the inhibition of calcium oxalate crystal growth and aggregation

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Background : *The inhibition of calcium oxalate monohydrate (COM) growth and aggregation can prevent the kidney stone formation. Black-purple rice (Oryza sativa), including Riceberry rice, contains high amount of antioxidant substances, such as anthocyanin, gamma - oryzanol and alpha-tocophorol. A previous study revealed that the high antioxidant content substances can prevent the formation of CaOx crystal.*

Objective : *To prove the high antioxidant activity of unpolished Riceberry rice extract can inhibit the growth and aggregation of CaOx crystal, especially COM.*

Methods : *Unpolished Riceberry rice (UBR) grain was extracted by ethanol. The monomeric anthocyanin content of the extract was assessed by pH differential method. The total phenolic compound was determined by Follin Ciocalteu reagent assay. The antioxidant activity assays were carried out by 1,1 diphenyl-2-picryl-hydrazyl radical scavenging activity assay and ferric reducing antioxidant power assay. The extracts were then incubated with the CaOx crystal, the number and the aggregation of CaOx crystal were then counted.*

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Results : *The results revealed that UBR extract contained high amount of anthocyanin content and possessed antioxidant activities. The co-incubation of the extracts and CaOx crystal could inhibit the growth of COM with dose-dependent pattern. Moreover, the aggregation of the CaOx crystal was significant reduced in the incubation with higher concentration of UBR extract.*

Conclusion : *This study indicated that UBR extract performed antioxidant activity, and could inhibit calcium oxalate crystal growth and aggregation. These results showed the high therapeutic potential of the UBR extract on the formation of kidney stone.*

Keywords : *Kidney stone, calcium oxalate, Riceberry rice, antioxidant.*

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วิทูร ขาวสุข, ทิษฏยา เสมาเงิน, ปริญญาพร หนูอุไร, วรเมธี มีปาน, เกียรติภูมิ วิงวอน. คุณสมบัติการต้านอนุมูลอิสระและผลการยังยั้งการก่อตัวและการรวมกลุ่มของผลึกแคลเซียมออกซาเลตด้วยสารสกัดข้าวกล้องไรซ์เบอร์รี่. จุฬาลงกรณ์เวชสาร 2561 พ.ค. – มิ.ย.; 62(3): 419 – 34

เหตุผลของการทำวิจัย : *การยังยั้งการก่อตัวและการเกาะเกี่ยวของผลึกแคลเซียมออกซาเลตโมโนไฮเดรต (COM) เป็นเป้าหมายในการควบคุมโรคนิ่วในไต จากการศึกษาข้าวที่มีสี รวมถึงข้าวไรซ์เบอร์รี่ พบว่ามีสารต้านอนุมูลอิสระในปริมาณสูง การศึกษาที่ผ่านมาพบว่าสารที่มีฤทธิ์ในการต้านอนุมูลอิสระสามารถยับยั้งการก่อตัวของผลึกแคลเซียมออกซาเลตได้*

วัตถุประสงค์ : *งานวิจัยในครั้งนี้มุ่งยืนยันว่าสารสกัดข้าวกล้องไรซ์เบอร์รี่ มีสารต้านอนุมูลอิสระสูงและสามารถยับยั้งการก่อตัวและการเกาะเกี่ยวของผลึกแคลเซียมออกซาเลตได้*

วิธีการทำวิจัย : *นำข้าวกล้องไรซ์เบอร์รี่มาสกัดด้วยเอธิลแอลกอฮอล์ แล้วนำมาศึกษาระดับแอนโทไซยานิน สารประกอบฟีนอล ความสามารถในการต้านอนุมูลอิสระ หลังจากนั้นนำสารสกัดมาผสมกับผลึกแคลเซียมออกซาเลต แล้วนับจำนวนผลึกที่เกิดขึ้น โดยเฉพาะชนิด COM และนับการเกาะเกี่ยวกันของผลึก*

ผลการศึกษา : *สารสกัดข้าวกล้องไรซ์เบอร์รี่มีแอนโทไซยานินและสารประกอบฟีนอลในระดับสูง และสามารถต้านอนุมูลอิสระได้ดี นอกจากนี้สารสกัดข้าวกล้องไรซ์เบอร์รี่สามารถยับยั้งการก่อตัวของผลึก COM ได้ตามความเข้มข้น ในขณะที่การเกาะเกี่ยวกันของผลึกลดลงเมื่อเพิ่มความเข้มข้นของสารสกัด*

สรุป : *การศึกษาครั้งนี้แสดงให้เห็นว่าสารสกัดข้าวกล้องไรซ์เบอร์รี่มีสารต้านอนุมูลอิสระในระดับสูง สามารถยังยั้งการก่อตัวและการเกาะเกี่ยวกันของผลึกแคลเซียมออกซาเลตได้ สารสกัดข้าวกล้องไรซ์เบอร์รี่จึงมีศักยภาพในการป้องกันการเกิดผลึกนิ่วในไต*

คำสำคัญ : *นิ่วในไต, แคลเซียมออกซาเลต, ข้าวไรซ์เบอร์รี่, สารต้านอนุมูลอิสระ.*

Nephrolithiasis is caused by the accumulation of calcium oxalate (CaOx) crystal in the renal tubule. Binding of CaOx crystal to the renal epithelium results in the alteration of membrane properties [1], destruction of mitochondria [2], upregulation of proinflammatory cytokines [3] and apoptotic factors [4], which leads to the unfortunately destruction of the renal epithelium. The supersaturation of urine, with the high concentration of oxalate and calcium molecules in the renal tubule, initiates the nucleation, growth and aggregation of CaOx crystal. CaOx monohydrate (COM), the most vulnerable subtype of CaOx, can bind the renal epithelium with a stronger manner and induced serious injury of cell than CaOx dihydrate (COD) and CaOx trihydrate (COT), respectively. [5, 6] Therefore, the investigation for blocking or diminishing the nucleation, growth and aggregation of COM or stimulation the transformation of COM to be COD and COT, the less vulnerable forms, will leads to the satisfaction therapeutic strategy.

The use of natural herbs for reducing the damage of renal epithelial cells has been increasing. Incubation the *Ammi visnaga* fruits extract with the MDCK and LLC-PK1, renal epithelial cell lines, resulted in lowering the damage of cell line caused by COM and oxalate molecule. [7] Epigallocatechin-3-gallate, green tea extract, showed the protective effects on the damage of MDCK cell line caused by oxalate molecule. [8] Antioxidative substances including vitamin E, apocynin, phycocyanin, fucoidin, gallotannins, rottlerin, lupeol and curcumin could perform nephroprotective property by down regulation of the oxidative stress production.[9,10] Moreover, the *Origanum vulgare* extract promoted the inhibition of COM nucleation and aggregation, reduced the toxicity of cell line caused by COM and prevented the kidney from the toxicity of CaOx deposition. [11]

Black-purple rice, including black sticky rice and Riceberry rice, contains numerous nutrients, such as, anthocyanin, gamma-oryzanol and alpha-tocopherol, which performed high antioxidant activities. [12, 13] It has been revealed that the anthocyanin-rich extract in the black sticky rice exhibited the reduction of oxidative stress and regulated the cholesterol homeostasis [14, 15], inhibited hemolysis induced by 2,2'-Azobis (2-amidinopropane) dihydrochloride and diminishes hydrogen peroxide production of mononuclear leukocytes. [16]

Unpolished Riceberry rice (UBR), purple color rice, is the most favorite strain for the health-concerned rice consumers. Interestingly, the extract of Riceberry rice whether contains substantial level of anthocyanin to inhibit the production of CaOx crystal formation. Therefore, this study aims to investigate the level of anthocyanin, antioxidant activities and the effects of unpolished Riceberry rice extract on the growth and aggregation of CaOx *in vitro*.

Methods

1. Preparation of UBR extract

UBR was ground to powder 2,000 g. The ground rice sample was extracted with 4,000 mL of 75% ethanol at RT for 24 hours with shaking. The extracted solution was filtered through no.4 filter paper. The sample was dried with rotary evaporator, kept at -20°C until use, and the yield of extraction was calculated before experimentation.

2. Determination of monomeric anthocyanin

Monomeric anthocyanin was determined by

pH differential method. Briefly, the 10% (w/v) extracted concentrate was prepared with 40% acetone. Determination of the appropriate dilution factor by diluting the test portion with 0.025 M KCl pH 1.0 buffer, until the absorbance at 510 nm is within the linear range of the spectrophotometer. Using this dilution factor, prepare two dilutions of the test samples, one with 0.025 M KCl pH 1.0 buffer and the other with 0.4M sodium acetate pH 4.5 buffer, determine the absorbance at 510 nm and 700 nm by using 40% acetone as blank. Absorbance was measured within 15 - 50 min of preparation. The absorbance of the diluted sample (A) was calculated as follows:

A = (A510nm – A700nm) pH 1.0 – (A510nm – A700nm) pH 4.5

The monomeric anthocyanin pigment concentration in the original sample was calculated by using the following formula:

Monomeric anthocyanin (mg/L) = A × MW × DF × 1,000/ε × 1 and it was converted to μg of total anthocyanin content per 1 gram rice grain.

Where MW is the molecular weight, DF is the dilution factor, and ε is the molar absorptivity, calculate pigment content as cyanidin-3-glucoside, where MW = 449.2 and ε = 26,900.

3. Determination of total phenolic compound

Total phenolic content of the extract was determined by Follin Ciocalteu reagent assay (Sigma). An aliquot 50 μl of gallic acid standard solutions (0.04, 0.08, 0.12, 0.16, 0.2, 0.24 and 0.28 mg/ml) and UBR extract solutions (0.2, 0.4, 0.6, 0.8, 1.0 mg/ml) were prepared. Each aliquot was added with 100 μl Follin Ciocalteu reagent and shaken. After 5 minutes, 1.5 mL of 5% sodium carbonate solution was added to the mixture and incubated at room temperature in the dark for 60 minutes. The absorbance against the reagent blank (40% acetone) was determined at 760 nm with spectrophotometer. Total phenolic content was expressed as mg gallic acid equivalents.

4. Ferric reducing antioxidant power (FRAP) assay

FRAP assay was determined by aliquot 100 μl of standard solution of ascorbic acid (0.2, 0.6, 1.0, 1.4, 1.8 mg/ml) and UBR extract solution (0.2, 0.6, 1.0, 1.4, 1.8 mg/ml). Each aliquot was added with 250 μl 0.2 M sodium phosphate buffer pH 6.6 and 250 μl 1% potassium ferric cyanide and mixed. After incubation at 50°C for 20 minutes, 250 μl of 10% trichloroacetic acid was added to the mixture and centrifuged at 2,200 g for 5 minutes. 250 μl supernatant was collected and added with 500 μl deionized water, 50 μl of 0.1% ferric chloride and vortex. The absorbance of the sample was measured against blank at 700 nm with spectrophotometer. The series of ascorbic acid standard solution were using as standard curve. The values obtained were expressed as mg of ascorbic acid equivalents.

5. 1,1 Diphenyl-2-picryl-hydrazyl radical (DPPH) scavenging activity

The percentage of antioxidant activity of UBR extract was assessed by DPPH scavenging assay. The samples were reacted with the stable DPPH radical in 40% acetone solution. An aliquot of standard solution of ascorbic acid (0.01, 0.02, 0.03, 0.04, 0.05, 0.06 mg/ml) and rice extract (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 mg/ml) were analyzed. The reaction mixture consisted of 0.1 mL of sample and 1.4 mL of 60 μM DPPH radical solution in 40% acetone.

The absorbance was read at 515 nm using a UV-VIS spectrophotometer after 30 min of reaction in the dark. The scavenging activity percentage was determined according to formula:

% Scavenging activity = (Ac – As) / Ac × 100

Where Ac = Absorbance of control, As = Absorbance of sample

The results were expressed as percentages of scavenging activity. The half maximal inhibitory concentration (IC_{50}) of both ascorbic acid and crude extract were reported.

6. The ability of UBR extract on the reduction of CaOx crystal formation

The calcium oxalate crystals were prepared in 24 well plate by adding 10 mM calcium chloride and 10 mM sodium oxalate, to reach the final concentration of 5 mM and 0.5 mM, respectively, with 1 mL of 10 mM Tris buffer containing 90 mM NaCl (pH 7.3). The triplicate reaction of 10, 20, 50, 100, 200 and 400 μg/ml of the UBR extract were added in the well. The mixtures were incubated overnight, RT, and observed under a phase contrast microscope. The average total numbers of CaOx crystal, COM (hexagonal shape), COD (octahedral shape) and COT (spindle shape) in each condition were random counted from 30 areas under high power field (40×) of phase contrast microscope.

7. The ability of UBR extract on the reduction of CaOx crystal aggregation

The calcium oxalate crystals were prepared in 24 well plate by adding 10 mM calcium chloride and 10 mM sodium oxalate, to reach the final concentration of 5 mM and 1 mM, respectively, with artificial urine pH 6.5. The triplicate reactions of 10, 20, 50, 100, 200 and 400 μg/ml of the extract were added in the well. The mixtures were incubated overnight, RT, and observed under phase contrast microscope. The average numbers of aggregated CaOx crystals in each condition were random counted from 30 areas under high power field (40×) of phase contrast microscope.

8. Statistical analysis

The results of triplicate reactions of all experiment were presented as mean ± SD and compared with the control group by using One-Way Analysis of Variance (ANOVA) with Turkey's post hoc test (SPSS ver.16.0).

Results

1. Total monomeric anthocyanin

The amount of monomeric anthocyanin per 1 gram of the UBR extract is 17.51 ± 6.89 mg, whereas, the calculated amount of monomeric anthocyanin per 1 gram rice grain is 2.45 ± 0.97 mg (Table 1).

Table 1. Showing the anthocyanin content of 1 gram UBR extract and 1 gram UBR grain

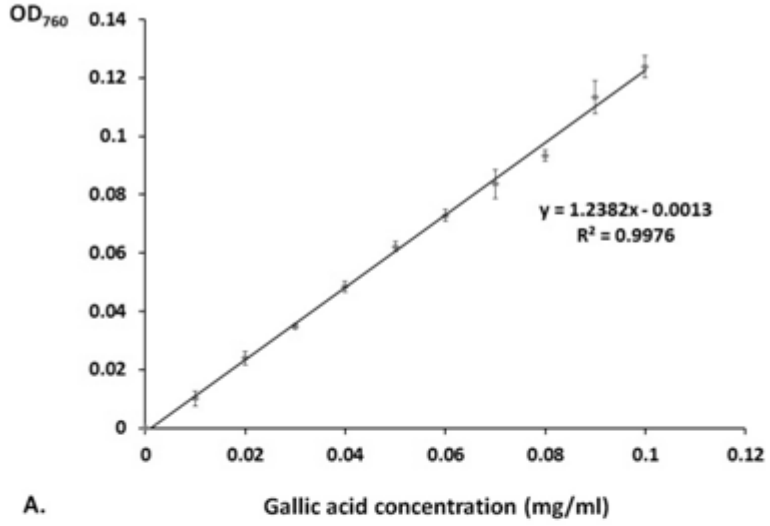
Yield of extraction (dry weight)	μg per 1 g extract	anthocyanin content μg per 1 g rice grain
1.4%	17.51 ± 6.89	2.45 ± 0.97

2. Total phenolic compound

The phenolic compound of gallic acid standard curve were plot, and the equation is y = 1.2382x - 0.0013 and R^2 = 0.9976 (Figure 1A). The total phenolic compound of the extract was calculated according to the equation of gallic acid standard. The phenolic compound of 0.2, 0.4, 0.6, 0.8, 1.0 mg/ml of UBR extract were 0.005 ± 0.000, 0.009 ± 0.002, 0.013 ± 0.002, 0.017 ± 0.001, 0.022 ± 0.002, 0.025 ± 0.002, 0.031 ± 0.003, 0.035 ± 0.002, 0.040 ± 0.003 and 0.043 ± 0.002 mg gallic acid, respectively (Figure 1B); the total phenolic compound per 1 gram of the UBR extract is 0.0240 ± 0.0131 mg gallic acid and the total phenolic compound per 1 gram UBR grain is 0.0221 ± 0.0011 mg gallic acid.

3. The percentage of DPPH scavenging activity

The scavenging activity of various concentration of ascorbic acid standard were 51.39 ± 0.40, 64.71 ± 3.05, 71.52 ± 1.26, 71.69 ± 0.15, 71.77 ± 1.04 and 72.57 ± 0.84, respectively, with IC_{50} about 0.01 mg/ml (Figure. 2A). The scavenging activity of various concentrations of UBR extract were 22.61 ± 3.33, 30.14 ± 5.03, 39.08 ± 2.61, 45.16 ± 3.51, 47.75 ± 3.87, 50.37 ± 4.63, 52.72 ± 1.80, 54.34 ± 0.23, 53.96 ± 0.18 and 53.60 ± 0.80, respectively, with IC_{50} about 0.60 mg/ml (Figure 2B).



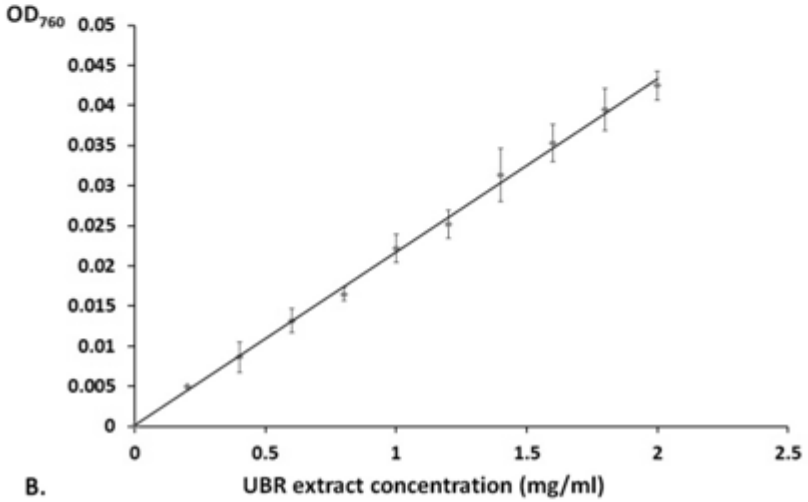


Figure 1. Total phenolic compound of gallic acid standard (A) and UBR extract (B).

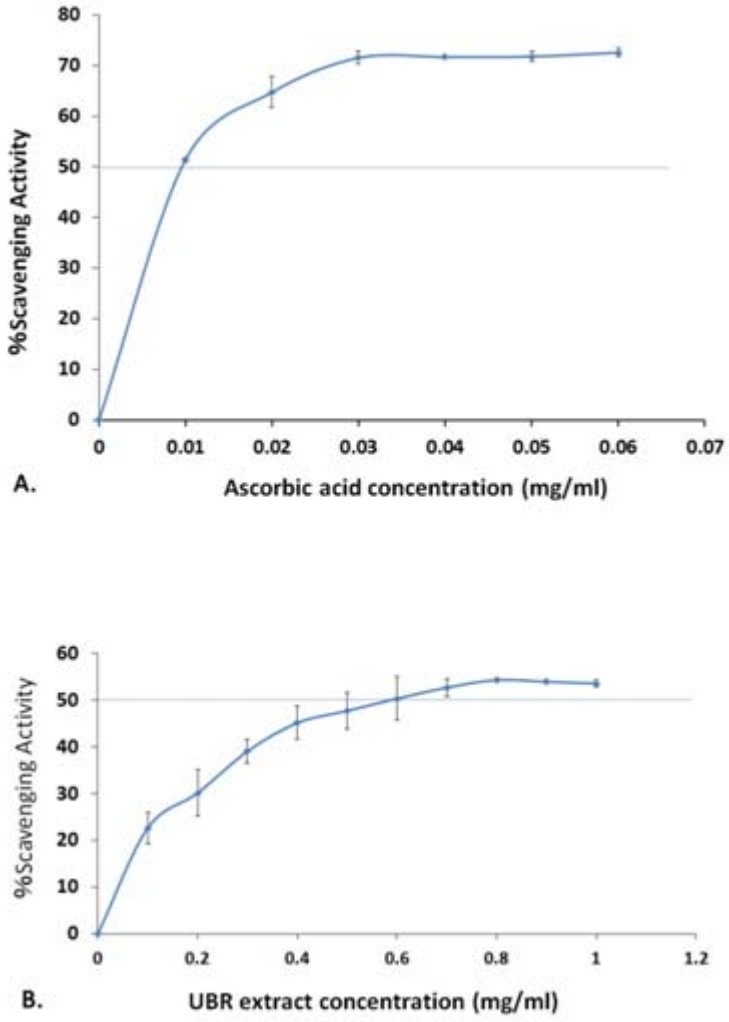


Figure 2. The DPPH scavenging activity of ascorbic acid standard (A) and UBR extract (B).

4. FRAP assay

The Fe^{2+} reduction activities of various concentration of ascorbic acid standard were 0.087 ± 0.015, 0.178 ± 0.013, 0.3 ± 0.008, 0.454 ± 0.023 and 0.618 ± 0.009, respectively, with the equation y = 0.3345x - 0.0072 and R^2 = 0.9875 (Figure 3A). The Fe^{2+} reduction activities of various concentration of UBR extract are 0.043 ± 0.013, 0.112 ± 0.005, 0.152 ± 0.001, 0.183 ± 0.012 and 0.214 ± 0.002 mg ascorbic acid, respectively (Figure 3B). The Fe^{2+} reduction activities of 1 gram of UBR extract was about 0.566 ± 0.087 mg ascorbic acid, whereas, the Fe^{2+} reduction activities of 1 gram of UBR grain was about 40.425 ± 6.223 mg ascorbic acid.

5. The effects of UBR extract on the CaOx crystal formation

The effects of UBR extract on the number of CaOx crystal were carried out at 0, 10, 20, 50, 100, 200 and 400 mg/ml body weight. The total number of CaOx crystal of the treated groups represented the significant difference from the untreated control group *($P < 0.05$)* at the concentration of 20 mg/ml, and the number gradually reduced with the increased

concentration (Figure 4A). The number of CaOx subtypes, COM (hexagonal shape), was reduced by the increased concentration of UBR extract. Whereas, COD (octahedral shape) and COT (spindle shape) were slightly increased in number in 10 mg/ml and 20 mg/ml treatment groups and showing highest number in the 50 mg/ml group, then decrease thereafter until 400 mg/ml (Figure 4B).

6. The effects of UBR extract on the CaOx crystal aggregation

The UBR extract at the concentration of 20 mg/ml showed a significant reduction of the number of aggregated CaOx crystal compared to the untreated group, and thereafter, the number gradually decreased with a dose-dependent manner toward 400 mg/ml (Figure 5).

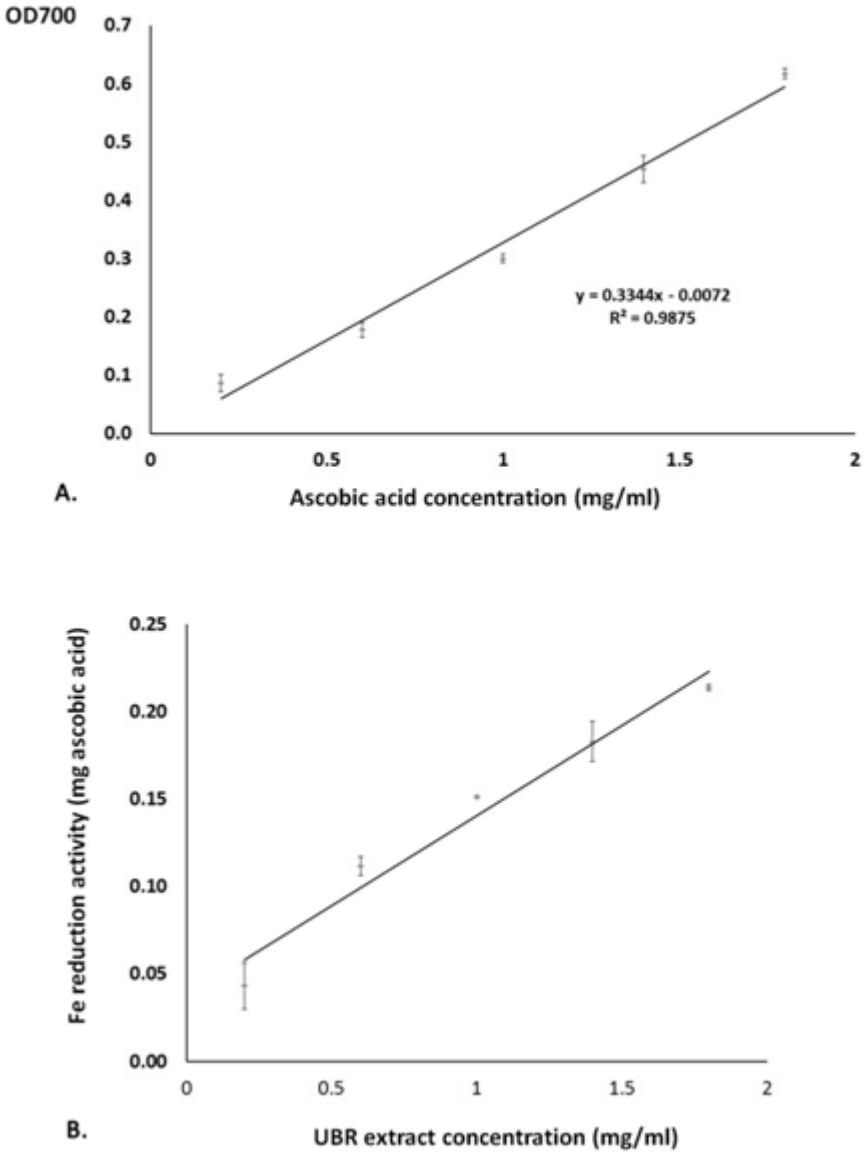


Figure 3. The Fe2+ scavenging activity of ascorbic acid standard (A) and UBR extract (B).

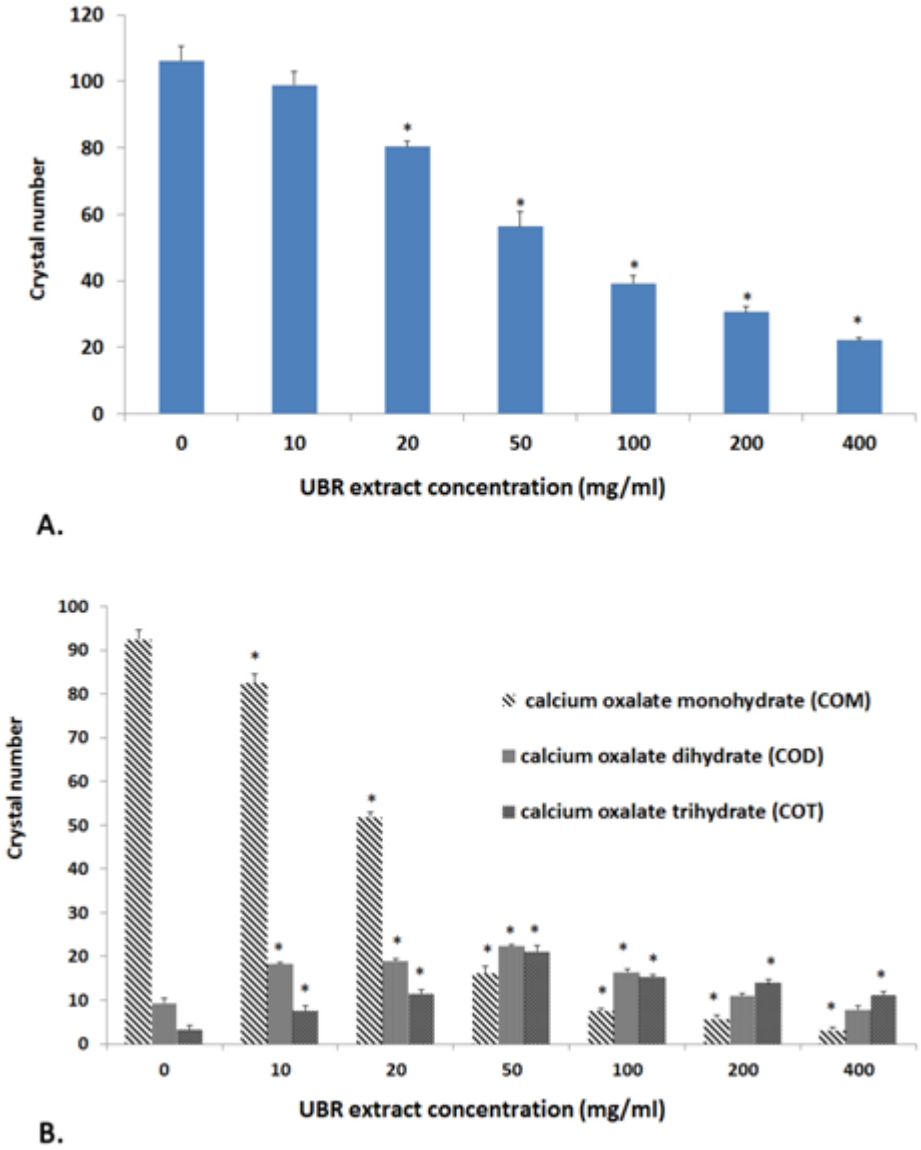


Figure 4. The effect of UBR extracts (0, 20, 50, 100, 200, 400 mg/ml) on the number of CaOx crystal in vitro showing the reduction of CaOx crystal number with dose-dependent manner (A). The effect of various concentrations of UBR extract on the number of COM, COD and COT (B).

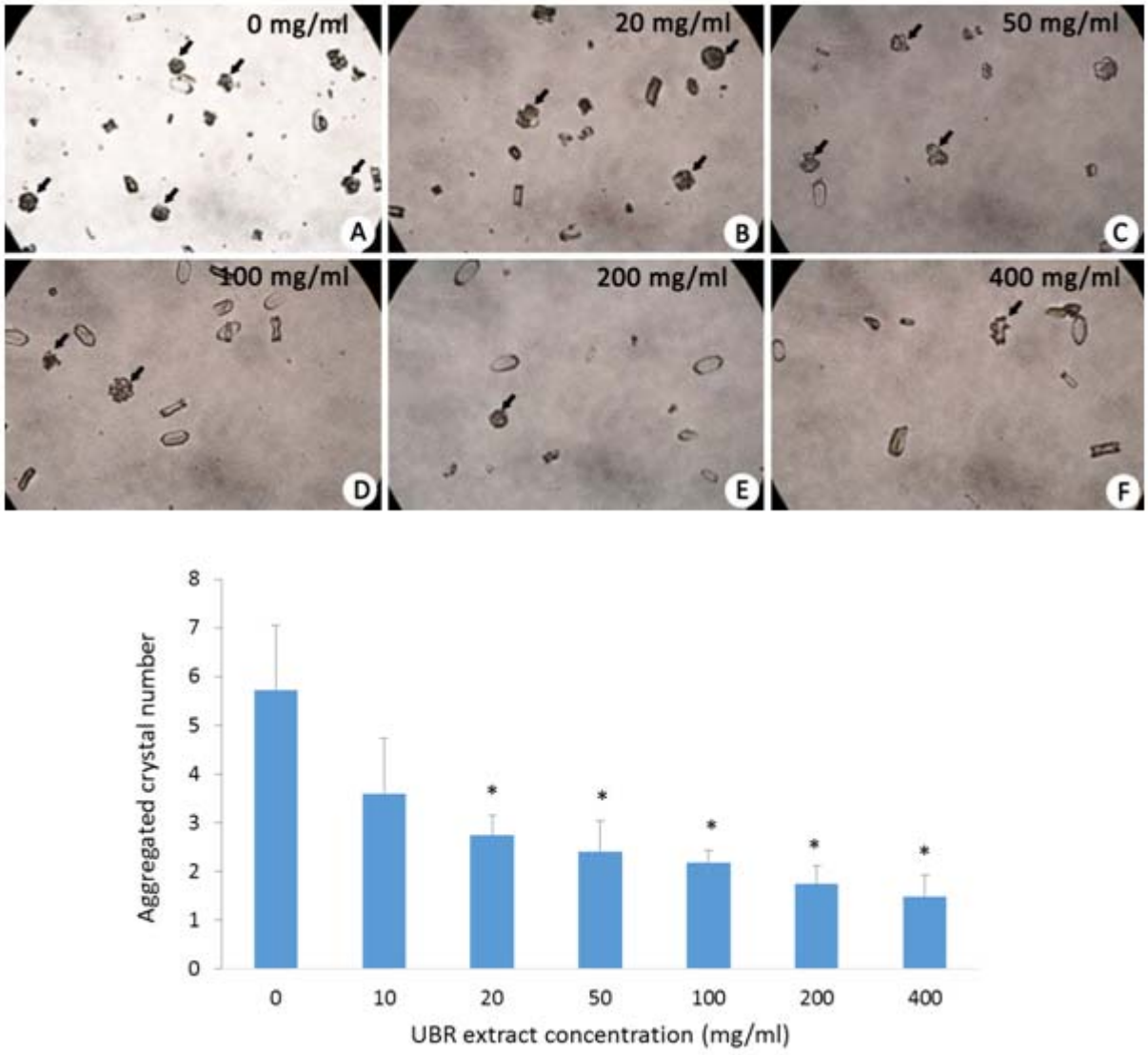


Figure 5. The effect of UBR extracts (0, 20, 50, 100, 200, 400 mg/ml) on the number of CaOx crystal aggregation (arrows in A-F) showing the reduction of the crystal aggregation with the dose dependent manner (G). 40x.

Discussion

This study is the first to demonstrate the effect of colored rice on the formation of CaOx kidney stone. UBR, nourished whole rice grain, was extracted by 75% ethanol to get black-purple oily substance. The anthocyanin content of UBR in this study is about 2.4 μg per 1 gram rice, which lower than that in black sticky rice, Hom Nil and Pitsanulok 2, respectively. [17] The lower amount of anthocyanin in UBR might be resulted from the thinner of pericarp, which possesses anthocyanin pigment. However, the amount of anthocyanin in black sticky rice is 96 times higher than corn and wheat and 35 times higher than red rice. [18]

Polyphenols compound can be roughly divided into non-flavonoid and flavonoid, which anthocyanin was classified as a member of flavonoid. [19] Therefore, the amount of total phenolic

compound in UBR extract might reflex the level of reactive substances, including anthocyanin. The content of phenolic compound of brown rice and white rice can be classified into the major insoluble and minor soluble form, whereas, phenolic compound of brown rice were more abundant than white rice. The increase of phenolic compound was observed in the germinated brown rice, especially ferulic acid and sinapinic acid. [20]

The antioxidant capacities of UBR extract have been elucidated by DPPH scavenging and FRAP methods. In agreement with the previous investigations, the present study showed that the oxidant scavenging activity in the colored rice extract is higher than that in the non-colored rice [21]. It has also documented that the black rice has higher oxidant scavenging activity than the red rice and normal rice, respectively. [22] The higher antioxidant activity of colored rice reflects the higher amount of anthocyanin content. The presence of anthocyanin, cyanidin 3-glucoside and peonidin 3-glucoside, of black rice leads to the marked preventing the DNA damage and suppresses the nitric oxide production by macrophage. [23] Long term storage of colored rice grain at room temperature lowers the antioxidant activity due to the fading of the anthocyanin overtimes. [24]

The protection of the kidney from the formation and the recurrence of kidney stone have been proposed by drinking large volume of water, alkalization urine by using oral potassium citrate, using pyrophosphate-containing solution for inhibition of stone formation and avoid high oxalate food consumption. [25] However, the calcium oxalate crystal formation in the kidney is governed by multiple factors, such as concentration of calcium and oxalate ions, concentration of urine, inhibitory factors in urine and pH of urine. [26] All factors involved in kidney stone formation are related to the diet consumption of the patients. Since, the use of in-house diets for protection the formation and the recurrence of kidney stone is of high potential.

Citrate is the calcium oxalate crystallization inhibitor, which found abundantly in the lime juice and sour fruit. The administration of patient with lime powder for 3 months resulted in the increase of urinary pH, reduction of lipid peroxidation of kidney and reduction of renal tubular damage. This was presumably due to the effects of lime powder in promoting the citraturic and alkalinizing urine, and elevating the level of antioxidative property of the kidney tissue. [27] After treatment the patients with lime power for 6 months, the total urinary proteins were decreased but the uromodulin, a urinary stone inhibitor, was increased. [28] According to the previous studies, the possible action of citrate for inhibition the calcium oxalate formation was binding to the calcium ion, and then stabilized the colloidal prenucleation form, thus prevented crystal aggregation and crystallization of CaOx. [29]

In the present study, UBR extract can reduce the total number and the aggregation of CaOx crystal with dose dependent pattern. These results show that the UBR extract has high potential in prevention of kidney stone. Although the mechanism of CaOx crystal inhibition by the UBR extract has been unproven in this study, the underlined mechanism might be from the antioxidant activity of a substantial level of anthocyanin in the extract. Anthocyanin, especially cyanidine-3-glucoside, has also been found

abundantly in the black-purple rice extract. [30] The molecular structure of cyanidine-3-glucoside is composed of two aromatic rings (A and B rings) bounded by oxygenated heterocycle three carbon atoms (C ring) and contains sugar residue at the C3 position. [31] This molecular structure indicates the antioxidative activity, which resulted from the chelating property of -OH residues of the C ring. [31] Therefore, anthocyanin, cyanidine-3-glucoside, of black-purple rice can sequestrate the metal ions, such as Cu, Fe or Ca, in the environment. In the condition of present experiment, the anthocyanin of UBR might take a chelating action on the Ca ion during incubation period, which leads to the reduction of CaOx crystal number and even the CaOx aggregation, similar to the action of citrate molecule.

Moreover, the incubation of CaOx crystal with a low concentration range of UBR extract (10 - 50 mg/ml) reduced the number of COM, while increased the number of COD and COT. These results possibly indicated that UBR extract might inhibit the formation of COM by reduced the number of CaOx precursor (Ca^{2+}), which leaded to the formation of COD and COT. Whereas, in the high concentration range (100-400 mg/ml), the total number of CaOx was reduced with dose dependent manner. This is hypothesized that the condition causes very low Ca^{2+} concentration which might not be enough to form CaOx crystal. However, this hypothesis is needed to be further elucidated by the measurement of Ca^{2+} concentration in the solution after the UBR extract incubation.

The previous *in vitro* and *in vivo* studies revealed that green tea extract [8], polyunsaturated fatty acid [32], polyphenol caffeic acid [33], vitamin E [10], low sodium diet, low calcium diet [34] and small molecules or magnetic field-applied water [35] could retard the kidney stone formation, and prevent the damage of tubular kidney cell caused by CaOx stone. Therefore, the protective effect of UBR extract against the kidney pathology caused by kidney stone has to be further investigated.

In conclusion, this study revealed that the UBR extract contained a substantial level of polyphenol and anthocyanin which can prevent the formation and aggregation of CaOx crystal, and the inhibiting activity might be from its antioxidant property. This study shows that UBR extract has a high potential for therapeutic use as the antiurolithiasic substance.

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