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In vitro Effect of Lipophilic and Hydrophilic Rice Strain Extracts in Sa Kaeo Province on the Prevention of Calcium Oxalate Crystal Formation and Aggregation

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ABSTRACT

Introduction: Kidney stone is a worldwide urological problem affecting human health, with a recurrence rate of up to 50%. It contains a multi-process involving crystal nucleation, growth, and aggregation. Using substances to interfere with these processes may be useful in reducing recurrent stone formation. The study aimed to investigate the inhibition effect of local colored rice strain extracts from Sa Kaeo Province on the formation and aggregation of calcium oxalate (CaOx) crystals *in vitro*

Methods: Black rice, black glutinous rice, and red rice strains were extracted using hexane and ethanol, called lipophilic and hydrophilic rice extracts, respectively. Rice extracts at different concentrations (10–400 µg/ml) were incubated with the CaOx crystal solution overnight. The number of CaOx crystals was counted and the percentage inhibition of CaOx crystal formation and aggregation was calculated. Statistical analysis with a significant difference was considered at $\alpha = 0.05$.

Results: All three rice extracts significantly decreased the number of CaOx crystal formation and aggregation. Using hexane extract, the red rice showed the highest inhibition of CaOx crystal formation, whereas the black glutinous rice showed the highest inhibition of CaOx crystal formation among the three rice strains' ethanol extracts. In addition, black glutinous rice had the strongest inhibition of the crystal aggregation among the three rice strains' hexane extracts, whereas black rice showed the highest inhibition among the three rice strains' ethanol extracts.

Conclusion: Black glutinous rice containing lipophilic and hydrophilic molecules was most effective in inhibiting the formation and aggregation of CaOx crystals. This study can be applied to the development of a supplemental product to prevent kidney stone formation.

Keywords: Lipophilic rice extract, Hydrophilic rice extract, Calcium oxalate, Crystal formation, Crystal aggregation

Introduction

Calcium stone is a cause of urolithiasis worldwide including in Thailand, especially in the Northeast region. The incidence of urinary stones in the 3 villages of Kon Kean Province detected by ultrasound was 16.9% and 2 times more abundant in males than females [1]. Most of this disease was affected by the deposition of calcium oxalate (CaOx) in parts of the uriniferous tubule [2, 3]. The internalization of calcium oxalate crystal by tubular epithelium resulted in the production of oxidative stress and led to the induction of renal inflammation process and fibrosis [4].

The calcium oxalate crystallization process is composed of nucleation, growth, and aggregation which are regulated by various factors. The hypercalciuria (>200mg/d) and hyperoxaluria (>40 mg/dl) [5] conditions resulted in the formation of calcium oxalate monohydrate (COM), calcium oxalate dihydrate (COD), and calcium oxalate trihydrate (COT). Moreover, kidney stone formation was modulated by urine acidity/alkalinity [6], concentration of citrate molecules [7], magnesium molecules, potassium molecules [8], and polyanion molecules [9].

Urolithiasis therapy by using herbal medicine is more promising than the limited-used pharmacological agents or the high recurrent rate extracorporeal shock wave lithotripsy or ureteroscopy treatments. The antilithiatic activity was proposed by using aqueous-extracted green tea [10], 70% ethanol-extracted avocado leaves [11], aqueous-extracted raspberry root [12], 99% ethanol-extracted roselle leaves, 70% methanol-extracted oregano [13] and aqueous-extracted *Herniaria hirsute* L. [14]. The 100% ethanol-extraction of *Acalypha indica* leaves expressed the highest inhibition effect on CaOx nucleation, growth, and aggregation over the chloroform-, ethyl acetate- and acetone-extraction [15].

Black rice and red rice were nourished with gamma oryzanol, alpha-tocopherol, tocotrienol [16], and anthocyanin [17], which contained powerful antioxidant capacity. Therefore, various strains of rice extracts were suggested for multiple health impacts using, such as antiaging [18], antiinflammation [19], anticholesterolemia [20, 21], anticancer [22], and antilithiasis [23, 24, 25]. The unpolished Rice Berry rice and local purple sticky rice bran extracted with 75% ethanol were shown to be the candidate agents by preventing the growth and aggregation of CaOx crystal *in vitro* [23, 25]. Moreover, unpolished Sungyod sticky rice, a local rice strain of Songkhla province, extracted with 75% ethanol could reduce the CaOx-induced kidney pathology by reducing free radical molecules and increasing antioxidant enzyme (SOD and catalase) expressions [24].

Purple rice bran was extracted with hexane and anhydrous methanol to obtain lipophilic and hydrophilic fractions. The majority of the lipophilic fraction was tocotrienol, tocopherol, and oryzanol, whereas cyanidine-3-glucoside was identified as a major constituent in the hydrophilic fraction. Moreover, the free radical scavenging capacity and total phenolic content of hydrophilic extract were higher than hydrophobic extract [26]. These studies demonstrated that the different solvents used in the rice research strategy resulted in diverse activities of the extracts.
Recently, the collection of various local colored rice strains from the Thailand-Cambodia border in Sa Kaeo Province was identified as the higher capacity free radical scavengers and high potential in cultivation promotion [27]. However, these colored rice strains are under agricultural farming research and cosmetic or pharmaceutical applications. Therefore, this study aimed to examine the inhibition effect of lipophilic and hydrophilic extracts of local colored rice strains collected from Sa Kaeo Province on the formation and aggregation of CaOx crystals *in vitro*.

Methodology

Preparation of rice samples

Three rice strains, namely black rice, black glutinous rice, and red rice were cultured on the organic farm at the Faculty of Agricultural Technology, Burapha University, Sa Kaeo Campus. 800 grams of each rough rice sample was milled at 2500 r/min for 5 min. The milled rice samples were then dried at 60 ^{o}C for 48 hr. The milled rice powder was stored at 4 ^{o}C until use.

Preparation of lipophilic extract

800 grams of each milled rice powder was extracted with hexane at a ratio of 1:5 and incubated in the dark for 24 hours. The extracted solution was filtered and collected in the test tube. The extract solution was then evaporated at 60°C and dried in a freeze dryer. The dried extract was weighted and calculated its lipophilic extraction yield.

Preparation of hydrophilic extract

800 grams of each rice powder were performed by the previous hexane extraction protocol. The residue was then dried and mixed with 75% ethanol at a ratio of 1:5 and incubated for 24 hours in the dark. The extracted solution was filtered and collected in the test tube. The residue was re-extracted once. The combined extract solution was then evaporated at 60°C and dried in a freeze dryer. The dried extract was weighted and calculated its hydrophilic extraction yield.

In vitro studies

(i) CaOx crystal formation assay

The CaOx crystal formation was prepared using the method of Thongboonkerd et al [28]. Briefly, the final concentration of 5 mM calcium chloride and 0.5 mM sodium oxalate solution was added in a 10 mM Tris and 90 mM sodium chloride buffer at pH 7.3. The extracts at the various concentrations including 10, 20, 50, 100, 200, and 400 µg/ml were prepared and then added to the CaOx crystal solution. As a control, the solution without any extract was performed. The reaction was incubated at room temperature overnight. The CaOx crystal formation was observed using a phase contrast microscope in a high-power field (40X) area. Ten areas/samples were used to calculate the number of CaOx crystals. The inhibition of CaOx crystal formation was analyzed as [(number of $CaOx_{control}$ - number of $CaOx_{extract}$)/number of $CaOx_{control}$] x 100.

(ii) CaOx crystal aggregation assay

The CaOx crystal aggregation was performed by the method of the Thongboonkerd et al. protocol [28]. In Brief, the mixture of calcium chloride and sodium oxalate solutions was prepared at the final concentration of 1 mM and 5 mM, respectively. The mixture solution was added into a buffer containing 10 mM Tris and 90 mM sodium chloride at pH 7.3. The extracts at the various concentrations including 10, 20, 50, 100, 200, and 400 µg/ml were prepared and then added to the CaOx crystal solution. The solution with no extract was used as the control. The reaction was then

incubated at room temperature overnight. The COM crystal aggregation was observed by a phase contrast microscope in a high-power field (40X) area. Ten areas/samples were used to calculate the number of CaOx crystal aggregations. The inhibition of CaOx crystal aggregation was determined using a formula: [(number of CaOx aggregated $_{control}$ - number of CaOx aggregated $_{extract}$)/number of CaOx aggregated $_{control}$] x 100.

Statistical analysis

The data was expressed as the mean and standard error of the mean (SEM). For multiple comparisons, the results were analyzed by one-way ANOVA, followed by Turkey-HSD's test. The significant difference was considered if P <0.05.

Results

Rice extraction yield

The yield of lipid content in red rice was slightly higher than that of black rice and black glutinous rice, whereas the yield of hydrophilic extract was the highest in black glutinous rice and the lowest in red rice (Table 1).

Table 1 Yields of the lipophilic and hydrophilic extracts from black rice, black glutinous rice and red rice

Rice extracts	Lipophilic extract	Hydrophilic extract
	(%)	(%)
Black rice	3.41	0.91
Black glutinous rice	3.53	1.96
Red rice	3.66	0.55

CaOx crystal formation after incubation with lipophilic and hydrophilic rice extracts

After incubation with lipophilic rice extracts at concentrations of 10, 20, 50, 100, 200, and 400 µg/ml. CaOx crystal formation was observed in all groups (Figure 1A). Black rice and red rice extracts tend to decrease the number of CaOx crystals with increased extract concentration, whereas high concentrations of black glutinous rice extract tend to increase the number of CaOx crystals (Figure 1B). The maximum percentage inhibition of CaOx crystal formation was 57.05±6.05, which was observed in red rice lipophilic extract at a concentration of 400 µg/ml.

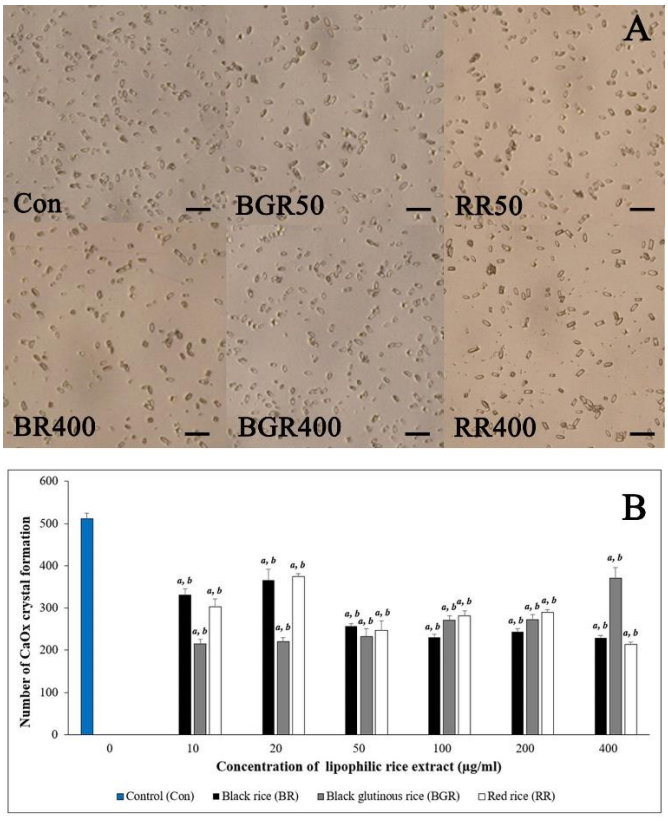


Figure 1 (A) The photograph showing CaOx crystal formation in the control group (Con) and lipophilic rice extract treated group at the concentration of 50 and 400 µg/ml (BR: Black rice; BGR: Black glutinous rice; RR: Red rice; scale bar = 10 µm). (B) The number of CaOx crystal formations in the control group (0 µg/ml) and lipophilic rice extract treated group (10-400 µg/ml). a and b indicate significant differences at p-value<0.05 and p-value<0.001, respectively compared

After incubation with hydrophilic rice extracts at concentrations of 10, 20, 50, 100, 200, and 400 µg/ml, all groups showed CaOx crystal formation (Figure 2A). Black rice extract tends to decrease the number of CaOx crystals with increased extract concentration. While black glutinous rice and red rice extracts at concentrations of 10 – 100 µg/ml significantly decrease the number of CaOx crystals. The number of CaOx crystals was increased when increasing the concentration to 200 and 400 µg/ml (Figure 2B). The maximum percentage inhibition of CaOx crystal formation was 71.37±9.18, which was observed in black glutinous hydrophilic rice extract at a concentration of 100 µg/ml.

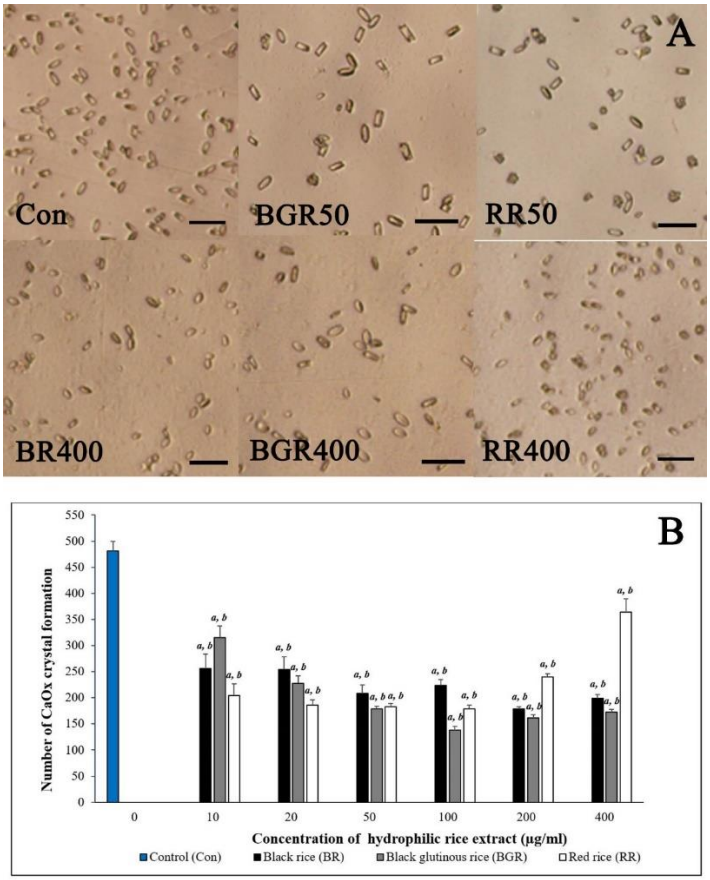


Figure 2 (A) The photograph showing CaOx crystal formation in the control group (Con) and hydrophilic rice extract treated group at the concentration of 50 and 400 µg/ml (BR: Black rice; BGR: Black glutinous rice; RR: Red rice; scale bar = 10 µm). (B) The number of CaOx crystal formations in the control group (0 µg/ml) and hydrophilic rice extract treated group (10-400 µg/ml). *a* and *b* indicate significant differences at p-value<0.05 and p-value<0.001, respectively compared to the control.

CaOx crystal aggregation after incubation with lipophilic and hydrophilic rice extracts

The CaOx crystal aggregation was identified in lipophilic and hydrophilic rice extracts (Figures 3A and 4A). The number of CaOx crystal aggregates was significantly decreased when increasing the concentration of all lipophilic rice extracts (Figure 3B). In addition, the highest percentage inhibition of CaOx crystal aggregation was 57.05±6.0, which was observed in black glutinous lipophilic rice extract at doses of 200 µg/ml. Following incubation with hydrophilic rice extracts, all rice extracts at concentrations of 10 – 100 µg/ml significantly decrease the number of CaOx crystal aggregations. The number of CaOx crystal aggregation was increased when the concentration increased to 200 and 400 µg/ml (Figure 4B). The highest percentage inhibition of CaOx crystal aggregation was 72.40±3.69, which was observed in black rice hydrophilic extract at a concentration of 100 µg/ml.

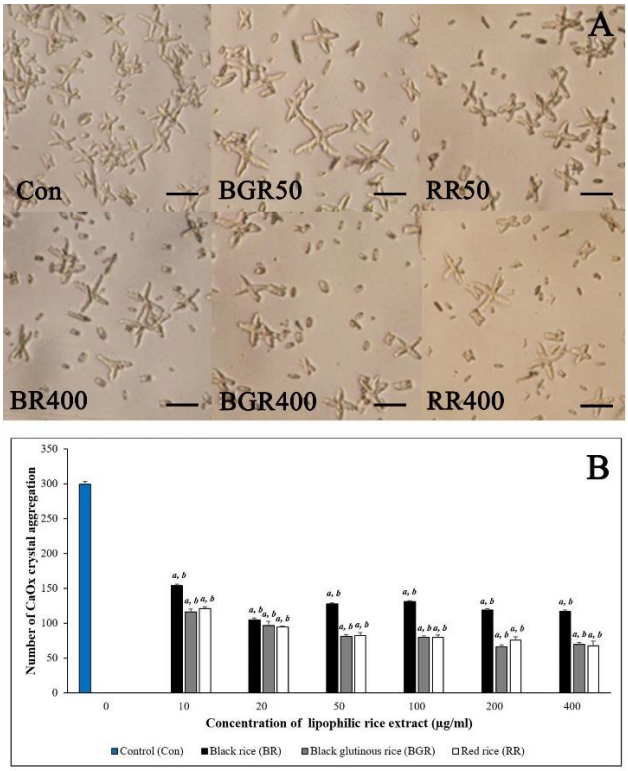


Figure 3 (A) The photograph showing CaOx crystal aggregation in the control group (Con) and lipophilic rice extract treated group at the concentration of 50 and 400 µg/ml (BR: Black rice; BGR: Black glutinous rice; RR: Red rice; scale bar = 10 µm). (B) The number of CaOx crystal aggregates in the control group (0 µg/ml) and lipophilic rice extract treated group (10-400 µg/ml). *a* and *b* indicate significant differences at p-value<0.05 and p-value<0.001, respectively compared to the control.

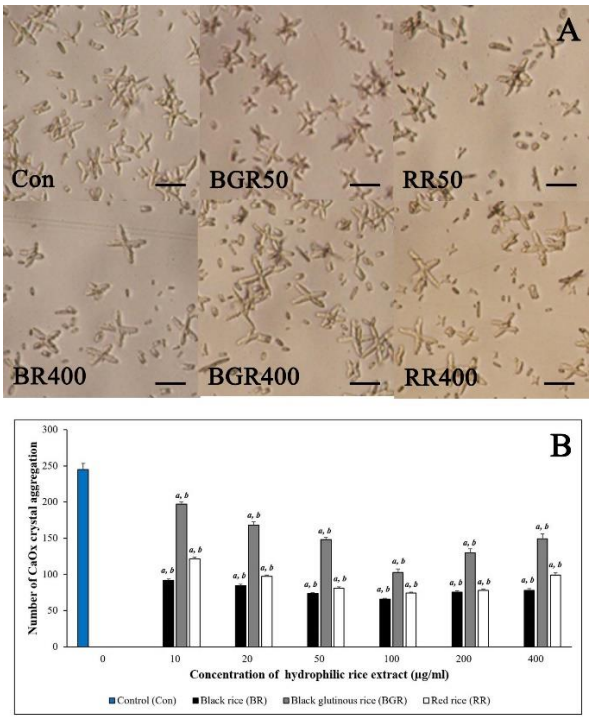


Figure 4 (A) The photograph showing CaOx crystal aggregation in the control group (Con) and hydrpophilic rice extract treated group at the concentration of 50 and 400 µg/ml (BR: Black rice; BGR: Black glutinous rice; RR: Red rice; scale bar = 10 µm). (B) The number of CaOx crystal aggregates in the control group (0 µg/ml) and hydrophilic rice extract treated group (10-400 µg/ml). *a* and *b* indicate significant differences at p-value<0.05 and p-value<0.001, respectively compared to the control.

Discussion

In this study, two solvents were used to extract three rice strains: black rice (non-glutinous rice), black glutinous rice, and red rice (non-glutinous rice). We also evaluated the percentage inhibition of CaOx crystal formation and aggregation compared to the control. These results showed that both extraction systems were able to detect the prevention of crystal formation and aggregation in all rice extracts with different degrees depending on the extraction solvent used. In this study, ethanol extracted tends to increase the percentage inhibition of CaOx crystal formation. In contrast, hexane extracted tends to increase the percentage inhibition of CaOx crystal aggregation. The result indicated that three rice extracts may contain different types and amounts of bioactive compounds that could differently affect crystal formation and aggregation.

Previous studies on the bioactive compounds in colored rice showed that purple rice bran contains lipophilic chemicals such as oryzanol, tocopherol, and tocotrienol, which could have antioxidant properties. Additionally, compared to the lipophilic extract, the hydrophilic extract of purple rice bran contained higher total phenolic components [26]. It was also comparable to the Peanparkdee et al. [29] showing that ethanol extract contained high amounts of phenolic acids, flavonoids, and anthocyanins. Previous studies also showed that pigmented rice such as red rice, black glutinous rice, and black rice contained a high amount of phenolic compounds and might be used as a natural antioxidant [25, 30].

Kidney stone formation is a multi-physiochemical process that involves crystal nucleation, growth, aggregation, adhesion to renal tubular cells, and internalization into renal epithelial cells [4]. The induction of CaOx crystal formation is a necessary step for subsequent crystal aggregation [31]. Previous studies have demonstrated that natural compounds like antioxidants and vitamins can interfere with the formation and aggregation of CaOx crystals. This interference may be useful in reducing recurrent stone formation [10, 32-36]. This study showed that black glutinous rice hydrophilic extract had a potential effect on the inhibition of calcium oxalate crystal formation in a low dose-dependent manner, while black glutinous rice and red rice lipophilic extracts had a potential effect on the inhibition of calcium oxalate crystal aggregation in a dose-dependent manner. These results correspond with a previous study on the local purple sticky rice bran extract. It showed that the extract at high concentration can induce CaOx crystal formation, especially COD crystals [25]. A previous study on *Herniaria hirsuta* extract showed that it can promote CaOx crystal nucleation, particularly COD crystals, by increasing their number while decreasing their size [14]. In this study, the morphology CaOx crystal was primarily present in the COM which appeared to be more harmful than COD due to their higher affinity for renal tubular cell damage [34]. It has been shown in a previous study that the formation of CaOx crystals depends on the charges of the ions in the solution. For example, proteins having acidic amino acid residues like Asp, Glu, and Gla will be deprotonated and negatively charged. They could interact with the positively charged Ca ions and inhibit the growth of CaOx crystals [37]. In addition, highly negatively charged proteins with Asp or Glu residues, like osteopontin, strongly bind to CaOx crystals and inhibit CaOx crystal formation, whereas weakly positively charged proteins, like Tamm−Horsfall protein, prothrombin fragment 1, albumin and bikunin, appear to primarily inhibit crystal aggregation and crystal adhesion to renal epithelial cells [38]. Accumulating studies suggest that several negatively charged macromolecules can interact throughout the crystallization process by modifying the shape or altering the electrostatic charge equilibrium of the CaOx crystals. [35, 38]. However, the exact molecule in natural substances including rice extract that affects the formation of CaOx crystals needs to be investigated further.

Conclusion

The potential for inhibition of crystal formation and aggregation in descending order was black glutinous rice, black rice, and red rice. The high content of lipophilic and hydrophilic active molecules in black glutinous rice could inhibit the formation of CaOx crystals as well as their aggregation. These findings suggest that colored rice may be used as a supplement to prevent the formation and aggregation of CaOx crystals. However, further studies are needed to determine how these extracts play a role in the prevention of kidney stone formation.

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