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Original article

Total phenolics, total flavonoid and total antioxidant capacity of medicinal plant-derived beverage: HydroZitLa

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Background: Antioxidants from natural sources are well-accepted for reducing oxidative damage and preventing the development of oxidative stress-related diseases. It is known that urinary stone development involves overproduction of reactive oxygen species and accumulation of oxidative injury. Supplements with antioxidants had been shown to inhibit kidney stone formation in animal models.

Objective: We developed a new beverage, called HydroZitLa (concentrate in pouch) to be used for prevention of urinary stone formation. HydroZitLa consisted of citrate (16 mEq) and naturally occurring antioxidants from Thai medicinal plants.

Methods: We investigated the total phenolic content (TPC), total flavonoid content (TFC), and total antioxidant capacity (TAC) in HydroZitLa. Also, cytotoxicity of HydroZitLa in HK-2 cells was evaluated.

Results: TPC and TFC in HydroZitLa were 10.2 ± 0.5 mg gallic acid equivalents (GAE) pouch and 2.8 ± 0.2 mg catechin equivalent (CE) pouch, respectively. TAC in HydroZitLa determined by 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)(ABTS) and 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) assays were 12.6 ± 0.2 and 11.3 ± 0.3 mg vitamin C equivalent antioxidation capacity (VCEAC) pouch, respectively. Stability test data at day 4 revealed that TPC and TAC (by DPPH) in HydroZitLa kept at 4°C were significantly higher than room temperature. Cytotoxicity test showed that HydroZitLa at 10% (v/v) and higher concentrations significantly caused reduction of HK-2 cell viability, and IC₅₀ of HydroZitLa was of 24.6% (v/v).

Conclusion: TPC, TFC and TAC in HydroZitLa were considerably high. Natural antioxidants together with citrate in HydroZitLa had a potential to prevent the formation of urinary calculi. Further pre-clinical and clinical studies should be conducted to warrant the therapeutic action of HydroZitLa in urolithiasis.

Keywords: Oxidative stress, antioxidants, HydroZitLa, citrate, urinary stone.

Oxidative stress is a condition with imbalance of reactive oxygen species (ROS) production and antioxidant capacity that potentially causes damage to cellular biomolecules leading to cell injury and death.⁽¹⁾ Oxidative stress mediates the development of several human diseases ranging from acute conditions such as infection to chronic conditions such as degenerative diseases and cancers.⁽²⁾ ROS

production and predisposition to oxidative stress are increased with age, therefore, regimens or approaches that are able to reduce oxidative stress have been suggested for prevention and treatment of oxidative stress - and age - related diseases.^(3, 4) Reduction of endogenous ROS production by caloric restriction and neutralization of the already formed ROS by antioxidants (especially natural dietary antioxidants) are well accepted for prevention of oxidative stress and oxidative stress-related conditions.⁽⁵⁾ Phytochemicals are naturally occurring compounds found in edible vegetables, fruits and medicinal plants, and many of them act as antioxidants.^(6, 7) Dietary polyphenols are the main class of these naturally occurring antioxidants, and several evidences have

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suggested a clinical role of the plant-derived polyphenols in disease prevention.^(8,9) Flavonoids are the largest group of polyphenolic antioxidants, and they have a benefit potential for improving human health.⁽¹⁰⁾ Banana stem (*Musa sapientum* L.), blue pea flower (*Clitoria ternatea* L.) and sappanwood (*Caesalpinia sappan* L.) are well-recognized as Thai traditional medicinal plants that contain high amounts of polyphenolic compounds and also flavonoids. Consumption of these medicinal herbs is usually in the form of drinks such as tea and beverages.

Urinary stone disease is the main urological problem worldwide, especially in Thailand.^(1, 11, 12) Based on our long research experience, we found that the most common stone type (up to 80.0%) was calcium oxalate stone, and there were three main causative factors, including inadequate water intake, low urinary excretion of citrate and high oxidative stress. Urinary stone disease is frequently recurred after stone removal. To date, the drug of choice for treating and preventing stone recurrence is potassium citrate. The drug mechanism of action is to increase urinary citrate and pH. Although the clinical efficacy of potassium citrate is generally accepted, it causes gastrointestinal side effects in some cases with low compliance rate^(13, 14), and unfortunately it has no antioxidant activity. We recently developed a new beverage called HydroZitLa (patent pending). It consisted of citrate (equivalent to the amount that used in potassium citrate drug) and water extracts from banana stem, blue pea flowers and sappanwood. Thus, the main active ingredients in HydroZitLa were water, citrate and naturally occurring antioxidants. It was clinically designed and developed to correct the three main causative factors of urinary stone formation.⁽¹⁾ We have proposed HydroZitLa beverage to be an alternative for urinary stone prevention with clinical efficacy comparable to the potassium citrate drug.

In this preliminary study, we aimed to measure total phenolic content (TPC), total flavonoid content (TFC) and total antioxidant capacity (TAC) in HydroZitLa and tested the stability of these antioxidant parameters within 5 days compared between storage at room temperature and in the refrigerator at 4°C. Also, cytotoxicity effect of HydroZitLa in human kidney cell line (HK-2) was investigated.

Materials and methods

HydroZitLa manufacture

HydroZitLa (patent pending) is an innovative beverage for reducing the risk of urinary stone

formation, developed at the Department of Biochemistry, Faculty of Medicine, Chulalongkorn University (led by C.B.). It was approved by Thai FDA in April 2019 (registered Thai FDA number: 10-1-02244-5-0005). HydroZitLa was manufactured by the GMP certified fruit juice factory (Jena Fresh Co, Ltd, Bangkok Thailand). It was produced in the concentrated form in sealed pouch (60 mL). For consumption, it had to be prepared by adding 500 mL of drinking water to HydroZitLa concentrate, mixing well and then be ready to drink. Amount of citric acid in HydroZitLa was 16 mEq/pouch. Antioxidants in HydroZitLa were derived from banana stem water extract (BSWE), blue pea flower powder (BPFP), sappanwood powder (SP) and alpha-lipoic acid (ALA).

Total phenolic content determination

Total phenolic content (TPC) in HydroZitLa was determined with the Folin-Ciocalteu's reagent (Merck, Germany) according to the method described earlier⁽¹⁵⁾ Briefly, Folin-Ciocalteu's reagent (200 µL) was placed into 96-well plate. Distilled water (blank), gallic acid standards (varied concentrations: 25, 50, 100, 200 and 400 µg/mL) and HydroZitLa (20 µL each) were added, mixed well and incubated for 5 min. Na₂CO₃ solution (2 M) (30 µL) was subsequently added and incubated at room temperature for 2 hours in the dark. Absorbance at 660 nm was measured. TPC in samples were calculated against the standard curve, and concentrations were expressed as mg gallic acid equivalent per milliliter (GAE/mL).

Total flavonoid content determination

Total flavonoid content (TFC) in each sample was determined by spectrophotometric method described earlier by Choi Y, *et al.* with minor modification.⁽¹⁶⁾ Briefly, distilled water (blank), HydroZitLa and standards (catechin at 0.031, 0.063, 0.125 and 0.25 mg/mL) (50 µL) was added to 320 µL of distilled water and mixed well. Sodium nitrite 5% (w/v) (15 µL) was then added, mixed well and incubated for 5 min. Aluminum chloride 10% (w/v) (15 µL) was added, mixed well and incubated for 6 min. Sodium hydroxide (1 M) (100 µL) was added and mixed we. Absorbance was measured at 510 nm. Concentrations of TFC were calculated against the standard curve and expressed as mg catechin equivalent per milliliter (CE/mL).

Total antioxidant capacity determination by ABTS and DPPH method

ABTS and DPPH assays were used for determination of total antioxidant capacity (TAC), and they were performed according to the previous studies with minor modifications. ^(17,18) For ABTS assay, 2.5 mM 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) was mixed with 1 mM 2,2-azobis(2-amidinopropane) dihydrochloride (AAPH) in 10 mM PBS (pH 7.4) and then heated at 68°C for 40 min. The obtained blue-green ABTS⁺ radical solution was cooled down. Absorbance (734 nm) of the radical solution was adjusted to 0.65 ± 0.02 with PBS prior to use. For the reaction, sample 5 µL was added to 295 µL of ABTS⁺ radical solution, mixed well and incubated at 37°C for 10 min. Absorbance at 734 nm was measured. Vitamin C at various concentrations (0.25, 0.5 and 1 mM) were used as standards to generate the standard curve. Level of TAC (by ABTS) was reported as mg vitamin C equivalent antioxidant capacity per liter (VCEAC/L). For DPPH method, 1 mM DPPH radical solution was prepared in 80% (v/v) methanol. Absorbance at 517 nm of the DPPH radical solution was adjusted to 0.65 ± 0.02 with 80% (v/v) methanol before use. For the reaction, the freshly prepared DPPH radical solution (295 µL) was placed into the wells of 96-well plate. Samples or vitamin C standards (5 µL) were added, mixed well and incubated at room temperature in the dark for 30 min. Absorbance at 517 nm was measured. Similar to TAC (by ABTS), level of TAC (by DPPH) was expressed as mg VCEAC/L.

Cytotoxicity assay

Cytotoxic effect of HydroZitLa in renal tubular HK-2 cells (purchased from ATCC, Manassas, VA, USA) was determined by MTT assay, HK-2 cells (200 cells/well) were seeded and grew in 96-well

plate overnight at 37°C, 5% CO₂ to achieve 70.0–80.0% cell confluence. Cells were treated with various concentrations of HydroZitLa (0 as control, 1.25, 2.5, 5, 10, 20, 40, 50, 80 and 100%, v/v) and then incubated at 37°C, 5% CO₂ for 24 hours. After discarding media, MTT solution (100 µL/well) was added and incubated for 1 hour. Cells were lysed by dimethyl sulfoxide, and the absorbance at 570 nm was measured. Cell viability (%) was calculated relative to control.

Statistical analysis

Data were presented as mean ± standard deviation (SD). Two-way repeated measures analysis of variance (ANOVA) was used to find the difference of antioxidant parameters between storages at room temperature and 4°C, followed by the Tukey's multiple comparisons test. GraphPad Prism 6.0 was used for all graphs and calculations. *P* < 0.05 was set as statistically significant.

Results

Total polyphenols, flavonoids and antioxidant capacity of HydroZitLa

The manufactured HydroZitLa was randomly sampled (n = 10) for measurements of TPC, TFC and TAC. The concentration and amount of TPC, TFC and TAC in HydroZitLa samples are shown in Table 1. Average concentrations of total polyphenols and flavonoids in HydroZitLa were 169.9 ± 8.3 µg GAE/mL and 45.9 ± 3.1 µg CE/mL, respectively. Average amount of TPC and TFC in one pouch of HydroZitLa were 10.2 ± 0.5 mg GAE/pouch and 2.8 ± 0.2 mg CE/pouch, respectively. Average amount of TAC in HydroZitLa determined by ABTS and DPPH assays were 12.6 ± 0.2 and 11.3 ± 0.3 mg VCEAC/pouch, respectively.

Table 1. Contents of total phenolics, total flavonoids and total antioxidant capacity in HydroZitLa.

Measurements	HydroZitLa (n = 10)
Total phenolic content	
Concentration (µg GAE/mL)	169.9 ± 8.3
Amount (mg GAE/pouch)	10.2 ± 0.5
Total flavonoid content	
Concentration (µg CE/mL)	45.9 ± 3.1
Amount (mg CE/pouch)	2.8 ± 0.2
Total antioxidant capacity (by ABTS method)	
Concentration (mg VCEAC/L)	210.3 ± 3.9
Amount (mg VCEAC/pouch)	12.6 ± 0.2
Total antioxidant capacity (by DPPH method)	
Concentration (mg VCEAC/L)	188.1 ± 4.5
Amount (mg VCEAC/pouch)	11.3 ± 0.3

Total polyphenols, flavonoids and antioxidant capacity of the main ingredients of HydroZitLa

The main raw materials used for manufacturing HydroZitLa were BSWE, BFPF, SP and ALA. To figure out the antioxidant contribution of each main ingredient in HydroZitLa, TPC, TFC and TAC were determined in BSWE, BFPF, SP and ALA samples at equal amounts that were used for making HydroZitLa (n = 10 for each). Table 2 shows contents of TPC, TFC and TAC (by ABTS and DPPH) in BSWE, BFPF, SP and ALA. ALA sample was undetectable for TFC, but it showed the highest content of total polyphenols. Based on the weight of raw materials, SP had the highest levels of TPC (4435 ± 275.8 mg GAE/100 g), TFC (3937 ± 47.5 μ g CE/100 g) and TAC (15753 ± 667.5 mg VCEAC/100 g by ABTS, 8707.0 ± 828.8 mg VCEAC/100 g by DPPH) compared to the others. Regarding amount added in HydroZitLa, BSWE had the highest levels of TPC (4.1 ± 0.3 mg GAE/pouch) and TAC (12.1 ± 0.9 mg VCEAC/pouch by ABTS, 9.7 ± 0.3 mg VCEAC/pouch by DPPH) relative to the other ingredients. It was indicated that TFC in HydroZitLa was mainly derived from SP (2.0 ± 0.1 mg CE/pouch), followed

by BFPF (1.9 ± 0.1 mg CE/pouch).

Stability of total polyphenols, flavonoids and antioxidant capacity in HydroZitLa

We preliminarily checked the stability of TPC, TFC and TAC in HydroZitLa for 5 days compared between storage at room temperature and 4°C. We found that keeping HydroZitLa at 4°C slightly preserved the content of antioxidants better than at room temperature (Figure 1). The only significant change was found at day 4, as the levels of TPC and TAC (by DPPH) in HydroZitLa kept at 4°C were significantly higher than room temperature (Figures 1A and 1D).

Cytotoxicity of HydroZitLa in renal tubular cells

Toxicity of HydroZitLa was tested in HK-2 cells using the standard MTT assay. Increased concentrations of HydroZitLa caused progressively reduced cell viability. At concentration of 10% (v/v) and higher, HydroZitLa significantly caused reduction of cell survival (Figure 2). The IC₅₀ of HydroZitLa in HK-2 cells was of 24.6% (v/v).

Table 2. Antioxidant contents of banana stem water extract (BSWE), blue pea flower powder (BFPF), sappanwood powder (SP), and alpha-lipoic acid (ALA) and their contribution of antioxidant contents in HydroZitLa.

Measurements	BSWE (n = 10)	BFPF (n = 10)	SP (n = 10)	ALA (n = 10)
Total phenolics				
Content in raw material (mg GAE/100 g)	8.1 ± 0.5	$1,296 \pm 89.7$	$4,435 \pm 275.8$	n/a
Content in HydroZitLa (mg GAE/pouch)	4.1 ± 0.3	3.2 ± 0.2	2.2 ± 0.1	n/a
Total flavonoids				
Content in raw material (μ g CE/100 g)	3.3 ± 0.1	745.6 ± 15.9	$3,937 \pm 47.5$	nd
Amount in HydroZitLa (mg CE/pouch)	1.6 ± 0.1	1.9 ± 0.1	2.0 ± 0.1	nd
TAC (by ABTS method)				
Content in raw material (mg VCEAC/100 g)	24.4 ± 1.7	$4,266 \pm 151.2$	$15,753 \pm 667.5$	$3,879 \pm 548.8$
Content in HydroZitLa (mg VCEAC/pouch)	12.1 ± 0.9	10.7 ± 0.4	7.9 ± 0.3	1.0 ± 0.1
TAC (by DPPH method)				
Content in raw material (mg VCEAC/100 g)	19.3 ± 0.7	$1,841.0 \pm 118.1$	$8,707.0 \pm 828.8$	$6,156.0 \pm 3,603.0$
Content in HydroZitLa (mg VCEAC/pouch)	9.7 ± 0.3	4.6 ± 0.3	4.3 ± 0.4	1.5 ± 0.9

nd: not detectable

n/a: not applicable

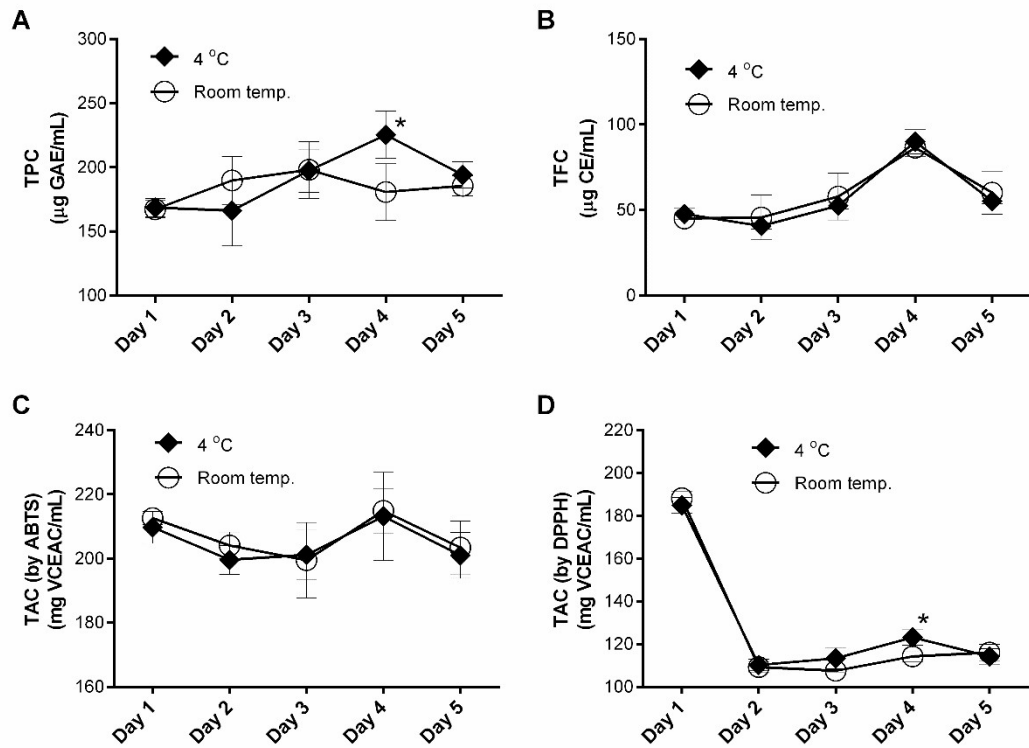


Figure 1. Stability of total phenolic content (TPC), total flavonoid content (TFC), and total antioxidant capacity (TAC, by ABTS and DPPH assays) in HydroZitLa compared between storage at room temperature and 4°C for 5 days. * $P < 0.05$.

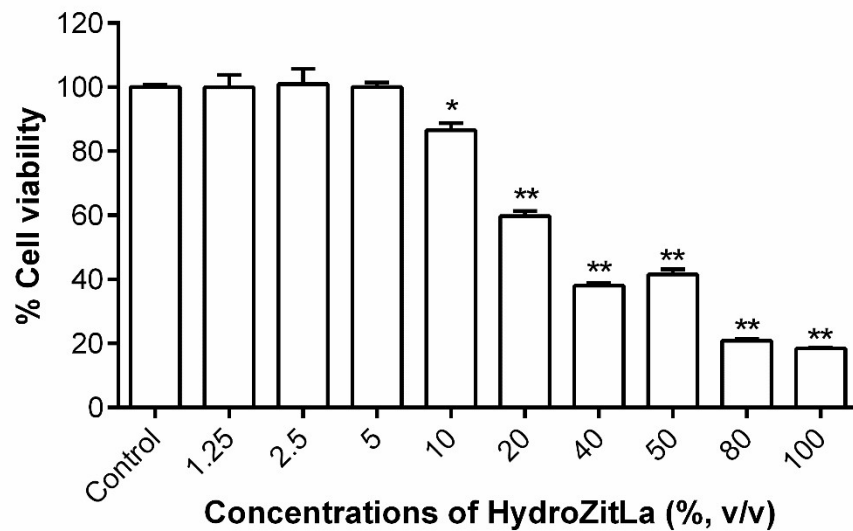


Figure 2. Cytotoxic effect of HydroZitLa in HK-2 cells assessed by MTT assay in 96-well plates for 24 hours. HydroZitLa at 10% (v/v) and higher significantly caused reduced viability of HK-2 cells compared to the untreated control. The IC_{50} of HydroZitLa in HK-2 cells was 24.6% (v/v). * $P < 0.05$, ** $P < 0.001$ vs. control.

Discussion

Increased production of ROS and depletion of antioxidants lead to oxidative stress that mediates pathogenesis of almost all chronic diseases including urolithiasis. (1,19,20) Our studies show that urolithiasis is prevalent in Thailand, particularly in the northeastern region, and the most common type of stones is CaOx with a trend of increasing prevalence of uric acid stone. (12, 21, 22) We also demonstrate that low urinary excretion of citrate (hypocitraturia) and potassium (hypokaliuria) are the main metabolic risk factors in Thai stone patients. (23, 24) As urolithiasis is an oxidative stress-mediated disease, antioxidant supplement has been shown to inhibit CaOx deposit in the kidneys of ethylene glycol-induced nephrolithic rats. (25, 26) Our human data showed increased urinary excretion and increased intrarenal expression of oxidative DNA lesion in patients with nephrolithiasis, indicating an enhanced oxidative stress. (27, 28) Antioxidants derived from fruits, vegetables, and medicinal plants rather than the commercially available antioxidant supplements have been recommended for the disease prevention, and it has been shown that increased intake of dietary fiber, fruits, and vegetables significantly decreases risk of kidney stone formation. (29) ALA is a well-known dietary supplement that has been shown to have clinical benefits on improvement of lipid profile, reduction of blood pressure and weight management. (30 - 32) ALA also has an antioxidant action, and it is shown to effectively reduce oxidative stress in human epidermal keratinocytes. (33) A recent study by Khawsuk W, *et al.* showed that the ethanol extract of unpolished Riceburry rice had high antioxidant activity and it could reduce CaOx crystal formation and aggregation *in vitro*. (34) We recently developed a novel beverage in the form of concentrate in pouch, called HydroZitLa (patent pending), containing citrate (a potent stone inhibitor) and water extracts of Thai medicinal plants. We had an intention to use it for prevention of urinary stone formation.

Herein, we measured the contents of total polyphenols, flavonoids and antioxidant capacity in the HydroZitLa product. Also, we determined the TPC, TFC and TAC in the main ingredients of HydroZitLa. We found that most of antioxidants constituted in HydroZitLa were derived from BSWE. The amount of TPC in HydroZitLa was 10.2 ± 0.05 mg GAE/pouch and amount of TFC was 2.8 ± 0.2 mg CE/pouch. Methanolic extraction of orange juice yielded TPC of 12.4 ± 4 mg GAE/100 mL (approx. 7.4 mg

GAE/60 mL). (35) Therefore, amount of TPC in HydroZitLa was roughly equal to 82 mL of orange juice methanol extract.

We checked the stability of antioxidant parameters in HydroZitLa compared between storage at room temperature and at 4°C in the refrigerator. We found that keeping HydroZitLa at 4°C slightly preserved the content of antioxidants better than at room temperature. Therefore, to lengthen the antioxidant quantity and quality of HydroZitLa, it was recommended to keep at 4°C in the refrigerator. The cytotoxicity of HydroZitLa was investigated in HK-2 cells. We observed that the significant cell toxicity was found at concentration of 10% (v/v) and greater concentrations. As the pH of HydroZitLa was around 2.9, we speculated that the toxic effect of HydroZitLa in cell culture model was might be due to the acidic pH of HydroZitLa. However, it should be noted some substances in the plant extract (that were not identified in this study) can also be responsible for the cytotoxic effect of HydroZitLa seen in this study.

Limitations of the study should be mentioned. This was only the preliminary study to determine the amounts of total phenols, flavonoids and antioxidant capacity in HydroZitLa. Therefore, extensive investigation on antioxidant efficiency in cell culture model did not explored. For the stability test, the storing period was relatively short (5 days). To be more informative about the shelf life of HydroZitLa the investigating storage time must be longer, e.g., several months.

Conclusions

We demonstrated that HydroZitLa had a relatively high antioxidant contents in terms of TPC, TFC and TAC (both by ABTS and DPPH methods). These antioxidant properties were principally derived from the medicinal plants, Banana stem (*Musa sapientum* L.), blue pea flower (*Clitoria ternatea* L.) and sappanwood (*Caesalpinia sappan* L.). Since HydroZitLa constitutes naturally occurring antioxidants together with citrate at therapeutic dose, it holds a promise for clinical use in prevention of urinary stone formation and recurrence. However, to verify the clinical efficacy of HydroZitLa intensive pre-clinical and clinical studies must be conducted.

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Conflict of interest

The authors, hereby, declare no conflict of interest. C.B., N.L., N.C. and N.M. are inventors of HydroZitLa.

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