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Effect of *Croton tonkinensis* Gagnep extract on calcium oxalate crystal formation causing kidney stones under *in vitro* conditions

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Abstract

The study investigated the use of *Croton tonkinensis* Gagnep extract to evaluate its ability to inhibit Calcium oxalate crystal formation, including three main stages: nucleation, growth, and aggregation. *Croton tonkinensis* Gagnep samples were extracted using a soaking method with 96% ethanol to obtain the concentrated extract. The percentage of inhibition of Calcium oxalate crystal nucleation by the *Croton tonkinensis* Gagnep extract was determined using spectrophotometry at a wavelength of 620 nm. Meanwhile, the inhibition efficacy on the growth of Calcium oxalate crystals was evaluated by measuring the optical density of the test sample at a wavelength of 214 nm over a period of 780 seconds. The inhibition efficacy on the aggregation of Calcium oxalate crystals by the extract was determined by measuring the optical density at a wavelength of 620 nm at different time intervals of 30, 60, 90, 180, 360, and 720 minutes. The research results showed that the extract had a high efficiency of 1.36%. The *Croton tonkinensis* Gagnep extract contained various compounds such as flavonoids, alkaloids, saponins, terpenoids, tannins, and phenols. The extract exhibited the ability to inhibit nucleation with an IC50 value of 7.78 mg/mL, as well as inhibit the growth and aggregation of Calcium oxalate crystals with times of 13 minutes and 90 minutes, respectively.

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Keywords: Calcium oxalate, kidney stones, aggregation, nucleus, growth, *Croton tonkinensis* Gagnep

1. Introduction

Calcium oxalate crystals are the main component of more than 60% of human kidney stones and exist in two main forms: Calcium oxalate monohydrate (COM) and Calcium oxalate dihydrate (COD). In the human body, kidney stones come in two sizes: small and large. Stones are small in size and can be excreted from the human body without affecting health. On the contrary, large stones can block the urinary tract, causing pain to the patient. In addition, medical measures are effective in treating Calcium oxalate stones but have a 50% risk of recurrence and many side effects such as damage to blood vessels in the kidneys and surrounding organs. (Tran Duc Tai, 2016) [1]. The current trend in the world and Vietnam is to research and develop natural products to support and treat diseases, because they have fewer side effects and are easier to find. Nguyen Pham Tuan and colleagues. (2020) evaluated the *in vitro* urinary stone inhibitory effect and isolated the active ingredient with urinary stone inhibitory effect of Pomegranate (Punica granatum) peel extract. As a result, pomegranate peel extract has the ability to inhibit nucleus formation, growth and condensation of Calcium oxalate crystals with IC50 values of 0.76 mg/mL respectively; 0.75 mg/mL and 0.99 mg/mL. Nguyen Quynh Chi and colleagues. (2015) evaluated the inhibitory effect of Calcium oxalate crystal formation from some species of the genus *Ficus* L. using the method of creating oxalate stones in artificial urine on a 96-well plate. According to folk experience, gentian leaves are used as a medicinal herb and have the ability to treat diseases. At the same time, gentian is a source of by-products that can be used to extract biologically active compounds for research and application in different fields.

The study was conducted to evaluate the effectiveness of medicinal plants in inhibiting the formation of Calcium oxalate crystals that cause kidney stones, aiming to create a source of raw materials for the production of products with medicinal properties to prevent and treat diseases.

2. Research content

2.1. Materials

Croton tonkinensis Gagnep plant material was obtained from Cu Chi district, city. Ho Chi Minh. Human urine samples were collected daily 2 hours before performing the experiment. Chemicals and equipment include: spectrophotometer, vacuum lyophilizer, Sodium oxalate ($Na_2C_2O_4$), Calcium chloride ($CaCl_2$) 0.01 M, Tris HCl pH = 6.5, Ethanol 96%, DMSO, DPPH 0.2 mM.

2.2. Research methods

2.2.1. Method for creating extract from gentian plant

A sample of 200 g of Gastroginseng plant was extracted and soaked with 96% ethanol, the ratio of raw materials and solvents is 1:10 (w/v), at room temperature 300C, for 48 hours and in dark conditions to limit oxidation process. Then the mixture will be filtered through filter paper to collect the filtrate and remove the solid part. The filtrate was then subjected to vacuum evaporation to remove the solvent and freeze-dried to obtain the extract from the Ginseng plant. After being collected, the extract was stored at -20oC for subsequent research.

Table 1: Identification of biologically active compounds in the extract of Gastroginseng plant

Compound name	Experiment	Phenomena
Akaloid	1 mL extract + Mayer's reagent	Brown precipitate
Flavonoid	1 mL extract + Pb(Oac)4 10%	Appears yellow
Saponin	3 mL extract + 6 mL -> boil	Foam appears
Steroid	1 mL extract + 2 mL CHCL3 + 2 mL concentrated H2SO4	Appears yellow red brown 2 layers
Tanin và phenol	0.5 mL extract + 10 mL H2O + 2-3 drops of 0.1% FeCl3	Dark blue precipitate
Terpenoid	2 mL extract+2 mL$(CH_3CO)_2O$ +2-3 drops of concentrated H2SO4	Appears dark red

2.2.2. Investigating the ability to inhibit the formation of Calcium oxalate crystals of the extract of Gentian ginseng

The method to investigate the ability to inhibit the formation of Calcium oxalate crystals of the extract of the Gastroginseng plant was carried out according to the method of Saha *et al*., 2013, improved according to the method of Phatak *et al*., 2015. Procedures were carried out. By thoroughly mixing 950 µl of nuclear sample with 100 µl of Gastrointestinal extract at different concentrations (from 0.25 mg/ml - 10 mg/ml) with the control sample being a sample without extract. Shake the mixture well for 2 - 3 minutes. Inhibition of Calcium oxalate crystal nucleus formation is calculated by the formula:

$$\% \text{ Inhibition} = \frac{(\text{OD Control sample} - \text{OD Extract}) \times 100}{\text{OD Control}}$$

In which: C: OD of control sample; S: OD of sample with extract

The positive control used to compare the effectiveness of inhibiting the formation of Calcium oxalate crystal nuclei with the gentian extract was Sodium citrate solution and was performed at the same concentration level as the extract. Evaluate the inhibition effect based on IC50.

2.2.3. Investigating the ability to inhibit the growth of Calcium oxalate crystals of the extract of Gentian ginseng

The method to investigate the ability to inhibit the growth of Calcium oxalate crystals of the extract of Gentian ginseng was carried out according to the method of (Chaudhary *et al*., 2010). The experiment was performed by adding 2 ml of urine sample, 1 ml of 0.01M $CaCl_2$, 1 ml of Tris HCl buffer pH=6.5 and 1 ml of 0.06M $Na_2C_2O_4$. Calcium oxalate is then added to the mixture to increase the reaction. The reaction between sodium oxalate and calcium chloride along with the nucleus crystal leads to the distribution of Calcium oxalate on the crystal surface. Let the reactions take place for 20 minutes, measure the absorbance at 214 nm to determine the initial level of free oxalate radical reduction, according to the time step of 3 minutes/time (4,7,10 and 13 minutes) and draw a graph to calculate the slope coefficient. Test sample: the reaction between Calcium chloride and Sodium oxalate takes place in 20 minutes, adding 1 mL of Gentian extract at different concentrations and measuring at wavelength λ= 214 nm. Measure the OD value every 3 minutes (4 minutes, 7 minutes, 10 minutes, 13 minutes) and plot the slope coefficient.

$$\% \text{ Inhibition} = \frac{\text{c-s}}{\text{c}} \times 100$$

C: inhibitor coefficient, S: control slope coefficient.

Assessed by spectral value and growth inhibition assessment chart.

2.2.4. Investigating the ability to inhibit the condensation of Calcium oxalate crystals of the extract of Gentian ginseng

Prepare the mixture according to the method of (Saha *et al*., 2013). The experiment was performed by adding 2 ml of urine sample, 1 ml of 0.01 M CaCl2, 1 ml of Tris HCl buffer pH = 6.5 and 1 ml of 0.06 M Na2C2O4. Incubate for 60 minutes to equilibrate and stabilize the crystals (no grow or dissolve in solvent) (Hess *et al*., 1989) and cool at 370C overnight. Testing the ability to inhibit COM crystal condensation of Gastroginseng extract according to the method (Saha *et al*., 2013). Dissolve in buffer including Tris pH = 6.5 and 0.01 M CaCl2 prepared at pH = 6.5, temperature 370C and final concentration of 1 mg/ml. Add 500 µl distilled water. Measure the absorbance of the control OD value without extract at λ = 620 nm at 30, 60, 90, 180, 360 and 720 minutes. Calcium oxalate crystal condensation inhibition reaction of gentian ginseng extract was performed at different concentrations. Add 1 ml of the extract at the concentrations and measure the absorbance at wavelength λ = 620 nm at intervals of 30, 60, 90 and 120 minutes.

$$\% \text{ Condensation inhibition} = \frac{(1-Si)}{Sc} \times 100$$

In which: Si: inhibitor slope; Sc: slope coefficient of control substance.
Evaluate by OD value and condensation inhibition assessment chart.

2.3. Data processing
The obtained data were subjected to descriptive statistics and analysis of variance (ANOVA), data were expressed as mean value±standard deviation, statistical comparison between the control group and the treated experimental group using Statgraphics Centurion 18 software with Tukey HSD method with 95% reliability. A p value < 0.05 was considered to represent a statistically significant difference.
Table graphs were drawn using Graphpad software

3. Results and Discussion
3.1. Qualitative results of gentian active ingredients

Table 2: Identification of biologically active compounds in the gentian plant

Tracking criteria	Result	Qualitative
Fresh sample weight (g)	200	Akaloid (+)
High dry volume (g)	2,72	Flavonoid (+)
Extraction efficiency (%)	1,36	Steriod (+) Tanin & Phenol (+) Saponin (+)

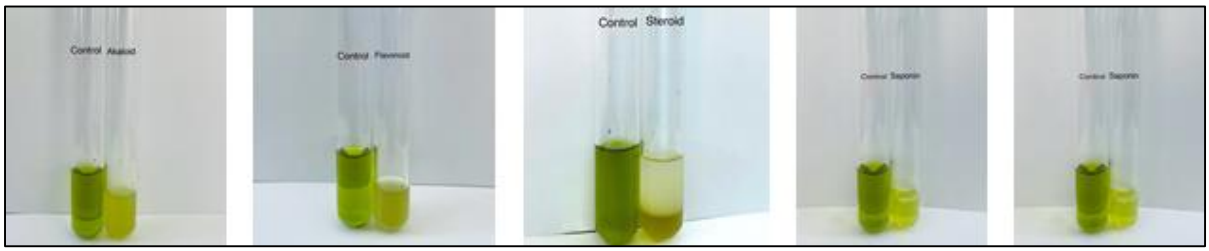

Fig 1: Identification of biologically active compounds in gentian

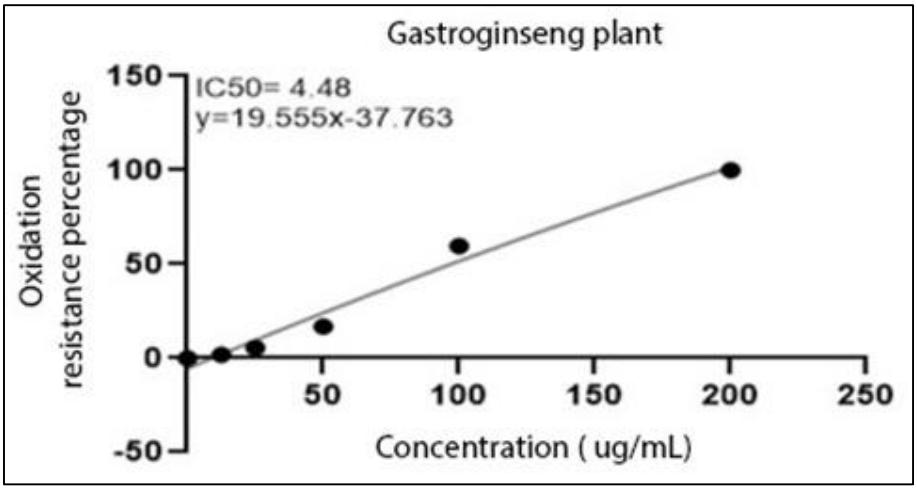

Fig 2: Oxidation resistance percentage chart of gentian gentian

3.2. Investigation of the ability to inhibit Calcium oxalate crystal formation of Gentian extract

Table 3: Results of measuring OD of Calcium oxalate crystal density across Sodium oxalate concentrations

Concentration Sodium oxalate (M)	Crystal density Calcium oxalate
Control	$0,001^{a}$±0,000
0.02	$0,25^{b}$±0,021
0.04	$0,51^{c}$±0,020
0.06	$0,61^{d}$±0,097
0.08	$0,65^{d}$±0,040
0.10	$0,61^{d}$±0,085

(a, b, c, d: Different letters in the same column indicate statistically significant differences at α=0.05; Data are presented as mean±standard error)

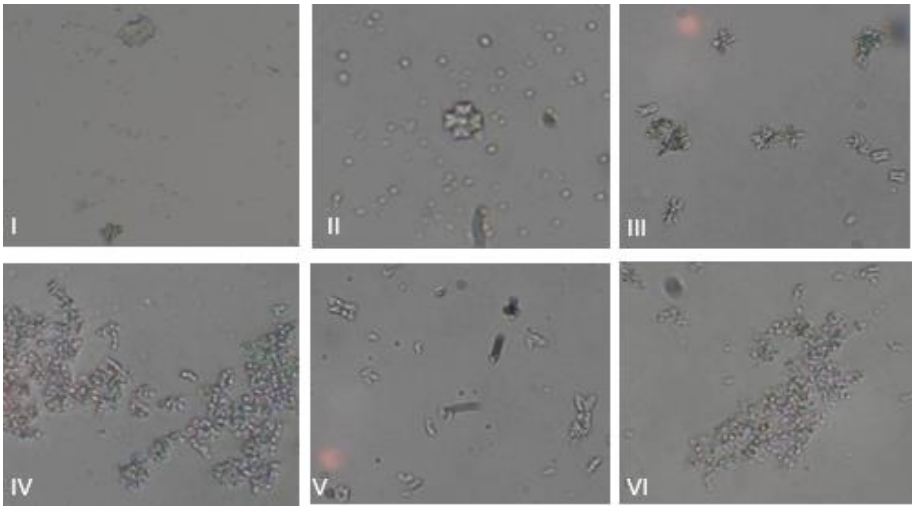

Fig 3: Calcium oxalate crystals through Sodium oxalate concentrations (40X) (I: Control; II: 0.02M; III: 0.04M; IV: 0.06M; V: 0.08M; VI: 0.10M)

Table 4: Results of percent inhibition of Calcium oxalate crystal formation by Gentian extract and Sodium Citrate

Concentration	Percent inhibition (%) of standard substance Sodium Citrate	Concentration	Percentage inhibition (%) of the extract of Gastroginseng plant
0	0^{a}	0	0
0.25	$28,61^{b}\pm7,83$	0.25	$36,19^{b}\pm3,48$
0.5	$34,50^{b}\pm2,97$	0.5	$34,32^{b}\pm25,37$
0.75	$53,90^{c}\pm2,03$	0.75	$37,30^{bc}\pm2,78$
1	$60,14^{cd}\pm2,27$	1	$35,55^{b\pm}2,47$
2	$62,76^{d}\pm6,77$	2	$41,78^{bcd}\pm2,78$
4	$75,47^{e}\pm3,52$	4	$37,88^{bc}\pm2,47$
6	$79,08^{e}\pm6,94$	6	$36,48^{b}\pm1,57$
8	$87,30^{f}\pm1,87$	8	$52,33^{cd}\pm4,22$
10	$94,81^{g}\pm0,86$	10	$56,53^{d}\pm11,17$
IC50	2,14	IC50 (mg/mL)	7,88

(a, b, c, d, e, f, g, h, k: Different letters in the same column indicate statistically significant differences at α=0.05; Data are presented. presented as mean± standard error)

Citrate salt compounds are substances that inhibit the formation of Calcium oxalate stones. They form complexes with Ca2+ ions, reducing the concentration of Calcium crystals. The effectiveness of inhibiting the formation of stone crystal nuclei through high concentrations of Gentian extract reached an inhibition effect of over 50% at a concentration of 8mg/mL with 56.53% inhibition and 10 mg/mL with 56.53% inhibition. 52.33%, above 30% has 0.25 mg/mL as the minimum concentration reaching 36.19%. With this result higher than Nguyen Pham Tuan *et al*. (2019), Purslane extract has inhibitory ability with IC50 = 3.69 mg/mL because COM crystals have a lower affinity for the cell membrane than COM crystals, so it is difficult to stick to the cell wall causing cell membrane damage. Kidney stones are easily eliminated through the urinary tract without causing damage. Whether COM crystals convert to COM or not decreases in density due to the presence of flavonoid compounds.

3.3. Investigating the ability of gentian extract to inhibit the growth of Calcium oxalate crystals

Table 5: OD measurement results to evaluate the survey of inhibiting the growth of Calcium oxalate crystals by extract of Gentian ginseng

Time / Concentration mg/mL	0 minute	4 minute	7 minute	10 minute	13 minute
0	0.095	0.043	0.125	0.085	0.054
0.25	0.03	0.02	0.07	0.082	0.017
0.5	0.055	0.027	0.027	0.08	0.007
1	0.061	0.051	0.061	0.114	0.035
2	0.046	0.06	0.064	0.112	0.048
4	0.068	0.099	0.121	0.171	0.067
8	0.066	0.086	0.079	0.128	0.048
16	0.07	0.034	0.038	0.067	0.012

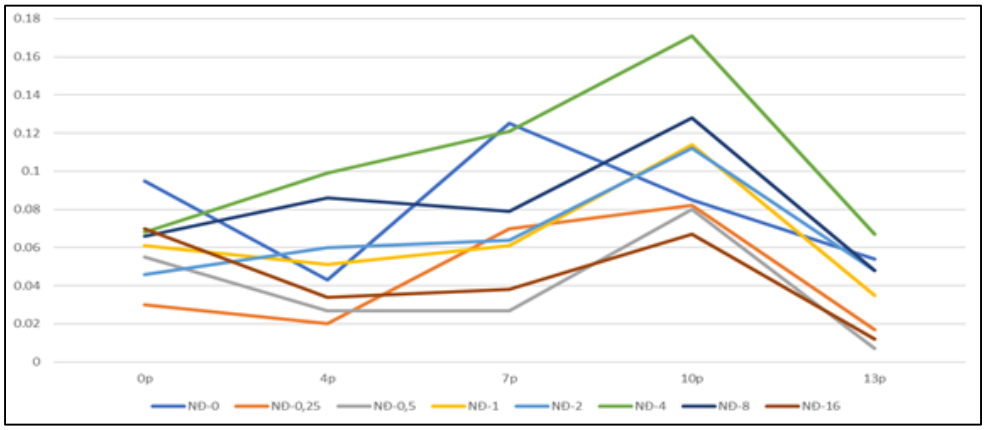

Fig 4: Assessment chart for surveying inhibition of the growth of Calcium oxalate crystals with ginseng extract

The effectiveness of inhibiting the growth of Calcium oxalate crystals is effective in a period of 13 minutes at a high concentration of Gastroginseng extract of 0.25 mg/mL; 0.5 mg/mL and 16 mg/mL are the same. Compared to the control, all concentrations inhibit the 13-minute time point. However, research results show that the 0.5 mg/mL concentration is effective the best. Curve showing the effectiveness of inhibiting the growth of Calcium oxalate crystals of the extract of Gentian ginseng.

3.4. Investigating the ability to inhibit condensation of Gentian extract on Calcium oxalate crystals

Table 6: Table of OD evaluation data showing the ability to inhibit the condensation of Calcium oxalate crystals by the extract of Gentian ginseng

Time / Concentration mg/mL	30 minute	60 minute	90 minute	180 minute	360 minute	720 minute
0	0.04	0.055	0.073	0.082	0.017	0.063
0.25	0.018	0.024	0.007	0.028	0.006	0.011
0.5	0.006	0.011	0.004	0.018	0.008	0.008
1	0.028	0.048	0.005	0.012	0.008	0.008
2	0.045	0.137	0.003	0.019	0.011	0.014
4	0.055	0.099	0.008	0.022	0.013	0.012
8	0.039	0.058	0.003	0.018	0.01	0.01
16	0.014	0.061	0.005	0.013	0.011	0.019

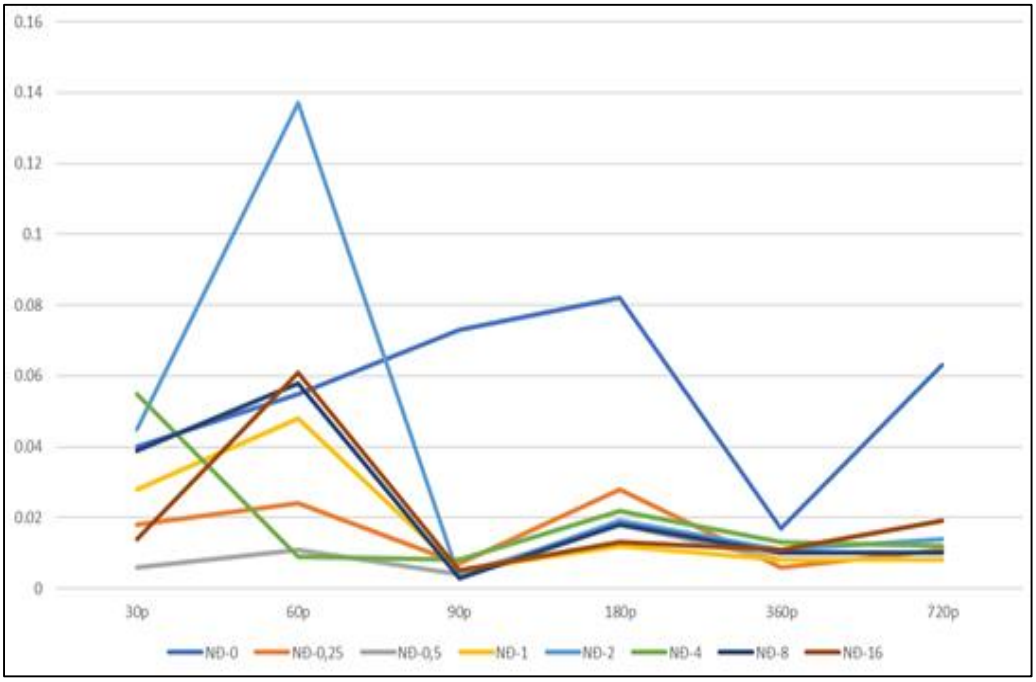

Fig 5: Chart to evaluate the condensation inhibition ability of Calcium oxalate crystals with ginseng extract

The effectiveness of inhibiting the condensation of Calcium oxalate crystals, all concentrations after 90 minutes decreased compared to the control. Most of the time, 180 minutes did not increase the effectiveness of inhibiting Calcium oxalate crystal condensation. However, a concentration of 1 mg/mL is the minimum concentration for the best results.

4. Conclusion

The gentian plant has alkaloid compounds, flavonoids, etc., which are the basic compounds of the group of medicinal plants. Research results show that the high extraction efficiency reached 1.36% Gentian extract has the presence of flavonoids, alkaloids, saponins, terpenoids, tannins and phenols. Gentian extract has the ability to inhibit nucleus formation with an IC50 value of 7.78 mg/mL, capable of inhibiting the growth and condensation of Calcium oxalate crystals for 13 minutes and 90 minutes, respectively.

References

1. Tuan NP, Lam BH, Tu NP, Le TN, Tai TD. Effect of Portulaca Oleracea L. on the formation of Calcium oxalate crystals causing kidney stones under *in vitro* conditions. Earth and Environmental Sciences; c2019. DOI: 10.15625/vap.2019.000223
2. Van TTH. Research on the chemistry and biological activities of some Vietnamese plant species in the direction of Vibrio paraheamolyticus strain resistance and cytotoxicity. Doctoral thesis in chemistry, Academy of Science and Technology; c2023.
3. Son TC, An TT, Binh VN, Lan DT, Quoc DD, Anh NT. Simultaneous analysis of 4 specific antihypertensive adulterants in herbal products using LC-MS/MS. Vietnam Journal of Food Control. 2023;6(2):176.
4. Thoan NV, Hung BM, Ha Ly NT, Phuong Thien Thuong. Developing a qualitative and quantitative method for the active ingredient ent-7β-hydroxy15-oxokaur-16-en-18-yl acetate in the medicinal herb gentian leaves (*Croton tonkinensis* Gagnep). VNU Science Magazine: Medical and Pharmaceutical Sciences. 2017;33(1):51-59.
5. Chewcharat A, Curhan G. Trends in the prevalence of kidney stones in the United States from 2007 to 2016. Urolithiasis. 2021;49(1):27-39. https://doi.org/10.1007/s00240-020-01210-w
6. Dhondup T, Kittanamongkolchai W, Vaughan LE, Mehta RA, Chhina JK, Enders FT, *et al*. Risk of ESRD and Mortality in Kidney and Bladder Stone Formers. American journal of kidney diseases: the official journal of the 29 National Kidney Foundation. 2018;72(6):790–797. https://doi.org/10.1053/j.ajkd.2018.06.012
7. Giang PM, Son PT, Lee JJ, Otsuka H. Four ent Kaurane-Type Diterpenoids from *Croton tonkinensis* GAGNEP., Chemical and Pharmaceutical Bulletin. 2004;52:879-882, 2004/07/10, Online ISSN 1347-5223, Print ISSN 0009-2363, https://doi.org/10.1248/cpb.52.879
8. Pham MQ, Iscache AL, Pham QL, và Gairin JE. Đặc tính gây độc tế bào, gây chết tế bào và mẫn cảm của diterpenoids loại ent -kaurane từ *Croton tonkinensis* Gagnep trên các dòng tế bào ung thư gan người HepG2 và Hep3b. Fundam 27 Clin Pharmacol. 2016;30:137-146. https://doi.org/10.1111/fcp.12176
9. Shekhar TC, Anju G. Antioxidant activity by DPPH radical scavenging method of Ageratum conyzoides Linn. leaves. American Journal of Ethnomedicine. 2014;1(4):244-249.
10. Yadav M, Chatterji S, Watal G. Preliminary phytochemical screening of six medicinal plants used in traditional medicine. International Journal of Pharmacy and Pharmaceutical Sciences. 2014;6(5):539-542.