Methanolic fraction of Malus domestica peel extract inhibits calcium oxalate crystallization in synthetic urine

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Abstract

Phytochemicals have been manipulated to largely contribute in the field of pharmacological studies. In this study, the peel extract of *Malus domestica*, more commonly known as apple, was evaluated for its anti-urolithiatic activity by inhibiting the formation of calcium oxalate crystals. More so, different solvents were tested in order to determine which could yield the highest flavonoid content in standard quercetin equivalent. To measure its inhibitory effects, the extract was tested in synthetically prepared urine samples. UV/Vis spectrophotometer was used to measure the nucleation and aggregation rate of the calcium oxalate crystals with the extract at 620 nm at various concentrations. The methanolic extract has the highest flavonoid concentration (77.9878 µg/100 g quercetin equivalent) among all the fractions tested in this study. Microscopic examination of spiked synthetic urine samples shows inhibition of calcium oxalate crystal formation with higher concentrations showing higher inhibition. Results of the study shows promising anti-urolithiatic action of the extract.

Keywords: *Malus domestica*, flavonoid, quercetin, calcium oxalate, potassium citrate

INTRODUCTION

Urolithiasis, a clinical condition involving the development of stones in the urinary tract, has been reported with an increased prevalence and of clinical significance (Chen, 2012). Considering the lifestyle and dietary choices of the majority, such is noted to contribute in the list of the most common health conditions (Yasui et al., 2017). In the study of Sohgaura & Bigoniya, (2017), Philippines is one of the countries with alarming occurrence rate of cases with renal

calculi in all age group including children below one year of age and adults over 70 years. However, most are asymptomatic which increases the chances of progressing to pyelonephritis and acute renal failure (Yasui et al., 2017). Renal calculi agglomerate in the urinary tract as a result of an increase in accumulation of crystal-forming substances which includes calcium, oxalate, uric acid and phosphates (Verkoelen& Verhulst, 2007). These crystals usually pass unnoticed in the urine however, some tend to adhere in the epithelial tubules. Sohgaura & Bigoniya, (2017) further explained that supersaturated urine induce crystal and stone formation. This is supported by Ratkalkar & Kleinman(2011) by enumerating the mechanism of stone formation which starts as the nucleation of stone constituent crystals, their growth to a size that can interact with the renal structure and their retention to the tubules or within the kidney. Further aggregation then leads to stone formation.

A number of clinical interventions like Extracorporeal Shock Wave Lithotripsy (ESWL) has become the standard method for eliminating renal stones thus the decline of open renal surgery (Yousif Mutalib Mc in Pharmacognosy & Yousif Mutalib, 2015). However, several drawbacks are also mentioned by Mc Ateer and Evan, (2008) which can occur following the procedure such as stone fragment residue, traumatic effect of shockwaves and possible infections. Some synthetic medicines are also available yet these are also costly and result in higher incidence of adverse medical reactions in comparison with the effect of herbal medicines. Thus, Chen (2012) stated that throughout history, a number of natural plant extracts are being utilized to improve stone passage and increase prognosis with little side effects. Several studies are continually being developed in search for plants with anti-urolithiatic activity In the study of Aggarwal, Singla, & Tandon, (2014) a list of plant species as anti-urolithiatic agents is drawn and the phytochemical which exhibits a significant decrease in the calculi number is identified with HPLC profile showing flavonoid quercetin as the major compound. Wientarsih, Madyastuti, Prasetyo, & Aldobrata, (2012) supported the claim in their study that showed the flavonoid property of extract of avocado leaves can prevent attachment of calcium oxalate crystals and stop the further formation of kidney stones.

Flavonoid is ubiquitously found in plantsand is associated with a broad spectrum of health-promoting effects (Panche, Diwan, & Chandra, 2016). Quercetin belongs to one of the sub-classes of flavonoid which is flavonols. In the journal of Li et al. (2015), it was reported that another sub-class of flavonoid,

catechin, mitigates nephrotoxicity and pathological renal disorder induced by melamine and cyanuric acid compounds. Thus, the search for plant with rich flavonoid content leads to the commercially- available and affordable, *Malus domestica*(Fuji apple). It is a main fruit crop in temperate regions of the world and is a member of family *Rosaceae*(Velasco et al., 2010). Apples contain a large concentration of flavonoids, as well as a variety of other phytochemicals. According to Feiman and Fine (2004), quercetin conjugates are found exclusively in the peel of the apples. Jha, Ramani, Patel, & Desai(2016) added that catechin is also abundantly found in apples. Health benefits of apples are already proven in several studies which include reduced risk for cancer, cardiovascular disease, obesity and diabetes. In vitro studies show antioxidant property, anti-proliferative activity and has cholesterol-lowering effects (Jha et al., 2016). Thus, this study aims to prove that the crude extract of *Malus domestica* can inhibit the crystallization of calcium oxalate in vitro.

MATERIALS AND METHOD

Collection and Identification of Plant material

Plant sample of *Malus domestica*(Fuji apple) waspurchased from the local market of Batangas and was taxonomically identified at the Bureau of Plant Industry, (See Appendix A).

Preparation of Plant extract

Malus domestica peelings were grinded in a low speed blender and each 10 g of the plant peelings were submerged in 75 ml of four different solvents namely ethyl acetate, petroleum ether, methanol and diethyl ether in a tightly sealed container for 48 hours. It was filtered simultaneously using a Whatman filter paper. The filtrate were evaporated for 3 days. Each yielded 100 mg of extract and was then measured for its total flavonoid content.

Malus domestica peelings were submerged in 80% methanol for 48 hours. It was grinded using a low speed blender and was filtered first throμgh a muslin cloth and then with a Whatman filter paper. The filtrate was concentrated using a rotary evaporator at water bath temperature of 60°C and vacuum for boiling point at 40°C for methanol in accordance to Sigma-Aldrich (2007).

Estimation of the Total Flavonoid Content

The total flavonoid content was estimated by aluminum chloride colorimetric method and based on the principle

stated Bag, Grihanjali Devi, & Bhaigyaba (2015). A 10mg of quercetin was dissolved in 100ml methanol and then diluted to 6.25, 12.5, 25, 50, and 100 µg/ml using methanol. Stock solution of plant extracts was prepared by dissolving 100 mg of the extract in 5ml of petroleum ether, diethyl ether and ethyl acetate respectively, transferred to 10ml volumetric flask and made up the volume with the solvent. 10% aluminum chloride and 1M potassium acetate were prepared using distilled water.

The assay was performed using 0.5ml of each extract stock solution and each dilution of standard quercetin taken separately in test tubes. To each test tube 1.5ml of each solvent, 0.1ml aluminum chloride solution, 0.1ml potassium acetate solution and 2.8ml distilled water were added and mixed well. Sample blank for all the dilution of standard quercetin and all the three extract were prepared in similar manner by replacing aluminum chloride solution with distilled water. All the prepared solutions were filtered through Whatman filter paper before measuring their absorbance. Absorbance was taken at 415 nm against the suitable blank.

Preparation of Synthetic urine

A 50ml of 2mmol/L of sodium oxalate was added to 50ml of 10 mmol/L of calcium chloride. The two solutions were prepared along with adding 9g of NaCl to obtain the ionic force like the indoor environment. According to the studies conducted by Barrett et al. (2012), the synthetic urine was prepared by mixing and stirring the two solutions and the sodium chloride salts at a constant temperature 37ºC in a capped vessel in order to give the final artificial urine. Mixture agitation was maintained to prevent sedimentation.

Light Microscopy study

Crystal count

The synthetic urine containing the controls and various concentrations was charged in a hemocytometer and was incubated for 30 minutes in 37ºC. The resulting calcium oxalate crystals were counted in all 25 RBCs squares under high power objectives (Aggarwal et al., 2014).

Spectrophotometric assay

Nucleation assay

The inhibitory activity of the extract that yields the highest flavonoid on the nucleation of calcium oxalate crystals was determined by a UV/Vis spectrophotometer based on Saha & Verma, (2013). Crystallization was initiated by adding

4mmol/L calcium chloride and 50 mmol/L sodium oxalate solutions to the synthetic urine, both prepared in a buffer containing 0.05 mol/L Tris and 0.15 mol/L sodium chloride at pH 6.5 maintaining 37°C. The rate of nucleation was determined by comparing the induction time of crystals (time of appearance of crystals that reached a critical size and thus become optically detectable) in the presence of the extract, that of the control with no extract and the positive control with potassium citrate. The absorbance was recorded at 620 nm, and the percentage inhibition calculated as (OD experimental/OD control)/100.

Aggregation assay

The same mixture of calcium chloride and sodium oxalate at 50mmol/L was prepared and equilibrated in a bath for 1 hour at 60°C. The solution was cooled at 37°C and evaporated. The calcium oxalate crystals were then dissolved with 0.05 mol/L Tris and 0.15 mol/L NaCl at pH 6.5 to a final concentration of 1 mg/mL. The absorbance at 620 nm was recorded every minute for 20 minutes. The rate of aggregation was estimated by comparing the slope of turbidity in the presence of the extract with that obtained in both negative and positive control. The percentage inhibition was calculated using the formula by Saha & Verma, (2013): % inhibition=1-((slope with inhibitor))/(slope without inhibitor)×100. The slopes of the nucleation and aggregation phases were plotted and calculated using linear regression analysis.

RESULTS AND DISCUSSION

Plant extract

For the extraction of the bioactive compound from *Malus domestica* peelings, organic solvents (methanol, ethyl acetate, petroleum ether and diethyl ether) were prepared for the comparison of the amount of flavonoid extracted. In the study of Sukri (2012), he stated that the ability of the solvents to diffuse easily to the pores of the plant materials is dependent on its viscosity. Low viscosity solvents have high diffusivity and low density which allow them to leach out the bioactive components.

After evaporation of the solvents, the extract from diethyl ether appeared as white, dry powder while the petroleum ether and ethyl acetate extracts appeared as yellow, dry powder. The methanolic extract appeared as viscous orange-brown syrup, all measured 100 mg (See Appendix B).

Estimation of total flavonoid content

The phytochemical screening was done by focusing on the total flavonoid content of the crude extract using four different solvents with increasing polarization. The aim is to obtain a crude extract with the highest flavonoid content even with the presence of other phytochemical compound. Methanol is the most polar among the four solvents, followed by ethyl acetate and petroleum ether. Diethyl ether is the least polar. It is measured using the Aluminum chloride colorimetric method (Bag et al.,2015).

Flavonoids are ubiquitously found in plants and are group of natural compounds with a variable phenolic structures. They have several subgroups and Panche et al., (2016) stated that a number of studies have proven its role in the pharmaceutical industry. The estimation of the total flavonoid content involves the use of quercetin as standard as it was commonly used to be a suitable reference for plant extract.

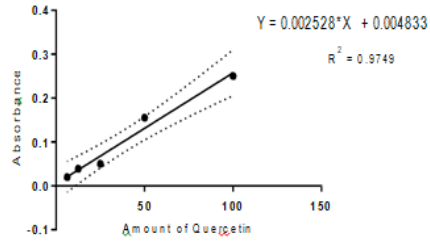

Figure 1. Calibration Curve of Absorbance (p= 0.0017)

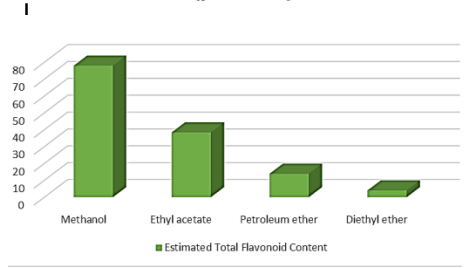

Figure 2. Estimated Total Flavonoid Content in µg quercetin/100mg extract

Figure 1 shows the standard calibration curve of quercetin which exhibited that the increasing dilution of quercetin prepared under the aluminum chloride colorimetric

method has an indirect effect on its concentration. The principle involved in the abovementioned method as discussed by Bag et al., (2015) is that aluminum chloride forms acid stable complexes with the C-4 keto groups and either the C-3 or C-5 hydroxyl groups of flavones and flavonols. In addition, it also forms acid labile complexes with the ortho-dihydroxyl groups in the A- or B-rings of flavonoids. A 100 µg/ml dilution of quercetin has an absorbance of 0.2500 at 415 nm. Increasing dilution of 50µg/ml, 25 µg/ml, and 12.5 µg/ml revealed 0.1550, 0.0500, and 0.0390, while the highest dilution of 6.25 µg/ml gave the lowest absorbance of 0.0200. The slope of the calibration curve is 0.9749 and has a p-value equal to 0.0017. In comparison with the solvents used, Figure 2 revealed that the methanolic extract has the closest absorbance to the highest concentration of quercetin. The results shown in Figure 2 are the concentrations of the solvents based on their absorbance at 415 nm as they were plotted in an analytical calibration using a simple linear curve fit.

Table 3 revealed that the methanolic extract that had the highest absorbance (0.2020) has an estimated flavonoid content of 77.9878µg quercetin/100 mg of extract. The extract yielded by ethyl acetate has an absorbance of 0.1020 and a calculated concentration of 38.4336 µg quercetin/ 100 mg of extract. Petroleum ether yielded a concentration of 4.949 µg quercetin/100 mg at the absorbance of 0.0400. Diethyl ether had the least absorbance (0.0150) and has an estimated flavonoid content of 4.0214 µg quercetin/100 mg of *Malus domestica* extract.

Table 3. Estimated Total Flavonoid Content of *Malus domestica*peel extract

Solvent	Absorbance	Total flavonoid content in µg/100 g of extract (in QE)
Methanol	0.2020	77.9878
Ethyl acetate	0.1020	38.4336
Petroleum ether	0.040	13.9099
Diethyl ether	0.015	4.0214

The coefficient of determination is equal to 0.9749 and y=0.0025x+ 0.0048 Similar studies had shown that the

methanol is the best solvent for the extraction of phenolic compounds in most plants. Stankovic (2010) studied the total flavonoid and phenolic content of plant extracts and concluded that high concentrations of phenols were yielded by the methanolic, acetone and water extract while petroleum ether and ethyl acetate extracts contain considerably smaller quantity of phenols. More so, among the three solvents with high concentrations, methanol yielded the highest concentration (1.98± 0.082). While in the research about the comparison of different solvents for phytochemical extraction potential by Dahwan and Gupta (2016) revealed that the maximum percent yield was from the methanolic extract, followed by the distilled water, acetone and acetate. Thus, these previous studies agreed with the results obtained from this study.

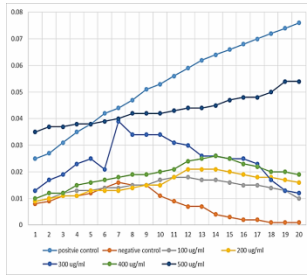

Figure 5. Plot of turbidity as influenced by different concentrations of *Malus domestica*extract and controls

The prevalence of urolithiasis is increasing worldwide and it is important to understand its pathogenesis. Kidney stone formation is initiated by the appearance of crystals termed as nucleation followed by their growth or aggregation and then their retention within the renal collecting system. Factors such as alteration of urine pH, decreased urine volume and supersaturation of stone-forming molecules can worsen the situation (Ratkalkar and Kleinman, 2012). Thus, the prevention of its development is essential in order to reduce the incidence of urolithiasis. Studies have shown that the most common crystals found in the urine and can induce stone formation are calcium oxalate crystals (Verkoelen and Verhulst, 2007). The formation of these crystals are without consequences as long as they are excreted in the urine. However, problem arises when crystal retention in the tubules occur.

Several prophylactic agents such as plant extracts and synthetic drugs have been developed throughout the years and clinical trials are continuously proposing agents for the inhibition of urolithiasis. In vitro assays are done in order to

evaluate if candidate drugs are capable of inhibiting these crystals. This study used spectrophotometric assay and microscopic crystal count to evaluate the inhibition property of *Malus domestica*methanolic extract against the synthetically formed urine. The absorbance of urine specimens with different concentration indicates its nucleation and aggregation. The initial absorbance of the controls and concentrations was a gradual increase which indicates the formation of the crystals upon the addition of calcium chloride and sodium oxalate and are optically detectable at 620 nm.

The negative control which is distilled water displayed nucleation as seen on its absorbance peak at 0.0160. 100 µg/ml had a peak absorbance of 0.0180, while 200 µg/ml, 300 µg/ml and 400 µg/ml exhibited their peak absorbance at 0.0210, 0.0390 and 0.0260 respectively. After the peak was reached, the absorbance declines as the particles start to aggregate. This downward slope of turbidity for the succeeding time indicates the phase of aggregation. The negative control and 100 µg/ml has an absorbance of 0.0010, while the concentrations 200 µg/ml, 300 µg/ml and 400 µg/ml had peaked down to 0.0160, 0.0120, 0.0190. The positive control, however had an exponential rise of absorbance which was also exhibited by 500 µg/ml concentration. This behavior showed that even if nucleation proceeded, the rate of aggregation was inhibited. The positive control had its peak at 0.0560 and continued to rise up to 0.0760 while the 500 µg/ml experimental group had a peak absorbance of 0.0440 and also exhibited a continuous increase of absorbance.

These correlate with the statistical analysis which showed that there was a significant difference between groups as determined by one-way ANOVA ($F_{6,133} = 91.226$, $p=0.000$). A Tukey post hoc test revealed that the absorbance was statistically significant between the positive control and the concentrations of 100 µg/ml, 200 µg/ml, 300µg/ml, and 400 µg/ml with a p-value of 0.000, while 500 µg/ml showed a p-value of 0.003 which was still considered as significant. However, there was no significant difference between the negative control and 100 µg/ml, 200 µg/ml, 300 µg/ml and 400 µg/ml.

The absorbance shown by the negative control and the concentrations from 100 µg/ml, 200 µg/ml and 400 µg/ml was increasing in the first phase which means that the nucleation process was not inhibited since nucleation is determined throµgh its direct relationship with the absorbance. Upon

attaining its maximum peak, the absorbance decreased as a result of the aggregation phase since it has an indirect relationship with the turbidity of the sample. In 300 µg/ml, it had its peak at 0.0290 and also had a downward slope. However, the 500 µg/ml extract showed a consistent rise of absorbance which means that aggregation of crystals was inhibited which is the same as the positive control.

The results represent the inhibitory effect of extract in correlation with the concentration. All the controls and experimental groups showed an initial increase in turbidity which denotes the increase formation of calcium oxalate crystals. This proved that *Malus domestica* peel extract has an anti-urolithiatic activity against calcium oxalate crystals if used in high concentrations. This agrees with the one of the Aggarwal et al., (2014) study that showed higher concentrations has better the effects.

Microscopic Assay

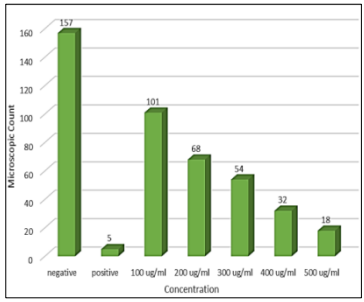

Figure 6. Microscopic Count of Different Concentrations

As shown on Figure 6, the number of calcium oxalate crystals seen in the negative control was 157 while the positive control decreased the count into 5 crystals. The extracts showed 101, 68, 54 32 and 18 crystals in 100, 200, 300, 400 and 500 µg/ml concentrations, respectively. Based on the results, the number of crystals seen varies from different concentrations of the extract. This may be due to several factors affecting the growth of crystals which include the presence of inhibitors which attributes to other phenolic compounds present in the extract. The concentration of such could also be involved in the inhibition factor affecting the crystals. Phytoconstituents are proven to have anti-urolithiatic properties which aids to the inhibition and crystal deposition (Sharma andJadhav, 2016).

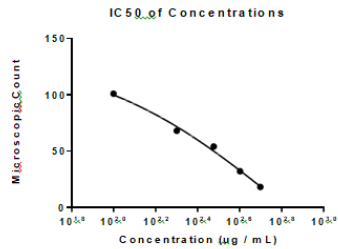

Figure 7. 50% inhibitory concentrations (IC50)

Figure 7. 50% inhibitory concentrations (IC50)

The microscopic results also showed a clear indication of crystal inhibition by *Malus domestica* peel extracts. The negative control was observed with numerous calcium oxalate with the highest count of 157 while the positive control with 5 crystals had the least count, both read on a Neubauer counting chamber on 25 RBC squares. The concentration of 100 µg/ml resulted almost the same as negative control with 101 crystals seen which means that the capacity of extract to inhibit the crystal formation is not noticeable. Crystals on 200 µg/ml were reduced into half having a result of 68 followed by 300 µg/ml which decreased the count into 54 crystals. These results shows that the increasing concentration of the extract had a notable effect on the inhibition of the formation of crystals. A significant decrease on calcium oxalate crystals was noticeably seen on extracts 400 and 500µg/ml having results of 32 and 18 crystals,respectively. Although, results may not be the same or as effective as the positive control, the number of crystals seen in concentrations 400 and 500 µg/ml may be evident that the methanolic extract has the capability to disrupt the calcium oxalate crystals.

The assay results showed that as the*Malus domestica* peel extract concentration increases, the inhibition value increases as well. Thus, a dose-dependent inhibition can be noted between the concentration of phytoconstituents and its properties (Agarwal &Valma, 2015). The phytochemical isolated on this fruit contains flavonoid which could be responsible for the observed antiurolithiatic potential. This is supported by the research done by Chilivery, Alagar and Darsini (2016) which showed the dose dependent increase in

the inhibition of calcium oxalate crystals of plant leaves extracted with methanol.

As shown on Figure 7, the relationship of the concentrations and its inhibitory property is further elaborated in the IC 50 (50% inhibitory concentration). The lowest concentration of the extract to inhibit 50% of the calcium oxalate crystal count was noted at concentration 500 µg/ml. Thus, a significant effect on inhibition property may be seen if higher concentration of phytochemical-riched extract was used. This is similar to the study conducted by Saha and Verma (2013) which showed the IC50 of their plant extract that inhibited the calcium oxalate crystallization at 0.9 mg/ml concentration.

Aside from crystal count, the appearance of calcium oxalate was observed on a different set of slides containing concentrations 100µg/mL to 500 µg/mL with the synthetic urine. As presented on Figure 8, calcium oxalate crystals were viewed using low- power objective with 100x magnification. The negative control (A) showed numerous calcium oxalate crystals against a clean field, while the positive control (B) presented a clean field as well with few crystals. The effectivity of potassium citrate was exhibited on this case due to its capability to dissolve the synthetically-made calcium oxalate crystals.

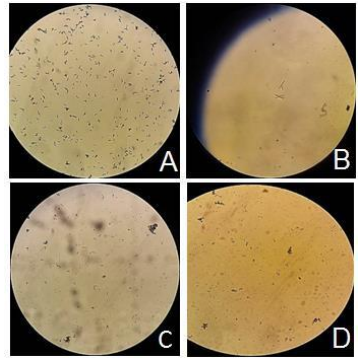

Figure 8. Calcium oxalate crystals observed on low power objective (100x magnification).
(A) as negative control, (B) as positive control, (C) as 100 µg/mL and (D) as 200µg/mL

Concentrations 100 µg/mL(C) and 200µg/mL (D) are almost similar to the field seen on the negative control. However, few clumps of the crystals were seen on both fields.

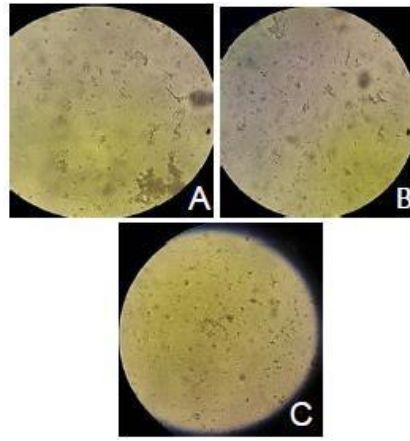

Figure 9. Concentrations 300 µg/mL(A), 400 µg/mL(B) and 500 µg/mL(C) observed on low power objective (100x magnification).

As shown on Figure 9, fields from concentration 300 to 500 µg/mL exhibits a dirty looking background with few counts of calcium oxalate crystals. The debris seen microscopically can be due to the disintegration of the crystals upon the addition of the methanolic extract. 500 µg/mL yields the least count of calcium oxalate in comparison with the other concentrations. Although it does not resemble the appearance of the positive control, a significant change was seen that may evidently show the effect of *Malus domestica*methanolic extract against calcium oxate crystallization.

Studies concerning the anti-urolithiaticbeffects of different plant extracts revealed that these phenolic compounds affect calcium oxalate crystallization. Chitra, Mothil and Tamizharasi (2017) focused on the *Pedaliummulex*and*Tribulusterrestris*plant extract in inhibiting calcium oxalate crystallization in vitro. Another study done by Shukla, Mandavia, Barvaliya, Baxi and Tripathi (2014) experimented on ethylene glycol-induced renal calculi in *Wistar* male rats for the evaluation of anti-urolithiatic effect of aqueous extract of *Bryophyllumpinnatum*. Such studies greatly contribute to the field of prophylaxis against the development of kidney stones.

CONCLUSION

Urolithiasis has been a world health threat for the past decades which led it to be one of the leading causes of kidney

diseases. Thus, a continuing research about phytotherapy against the said disease is conducted. This study focused on assessing the inhibitory effect of *Malus domestica* peel extract which contains flavonoids, against calcium oxalate crystals. The estimation for the total flavonoid content showed that methanol was the best solvent in extracting the said phytochemical. However, presence of other phytochemicals are to be considered since the extract is not purified. The spectrophotometric absorbance revealed that the positive control (potassium citrate) still had the highest inhibitory effect on the crystals. However, among the experimental groups, 500 µg/ml concentration had shown a promising inhibitory action. In the microscopic analysis, the number of crystals observed decreased as the concentration increased. This proved that *Malus domestica* can be used as a phytotherapy against urolithiasis.

RECOMMENDATION

The researchers recommend further isolation of the flavonoid extract in a purified concentration. In vivo experimentation for supplementary determination of *Malus domestica* extract effect on calcium oxalate crystals is also recommended as well as a comparative study between different cultivar of apples to determine the clinical effectivity of each on urolithiasis.

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