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In Vitro Antilithiasis Activity of Melastoma malabathricum Linn

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Abstract
Urolithiasis is a painful disease that affects the human population in every part of the world and majority of cases of renal lithiasis have calcium oxalate. The present study was carried out to study effect of *M. malabathricum* on calcium oxalate crystals *in vitro* and to determine the relationship between total flavonoid content and anti-crystallization activity of different extract. Four different extract from different parts of *M. malabathricum* were studied to determine the part with higher activity against calcium oxalate crystallization.The leaf of *M. malabathricum* demonstrated the highest crystal growth inhibition activity compared to other parts of the plant. The leaf extract was further fractionated by column chromatography. The fractions were studied for total flavonoid content as well as crystal growth inhibition and Spearman correlation showed that there was a good correlation between TFC and antilithiasis activity with the F1 fraction having highest activity. The activity of the F1 fraction was confirmed by calcium oxalate aggregation assay and microscopic examination. The higher content of flavonoids and calcium oxalate antilithiasis activity of the leaves suggest further studies for development of newer effective drugs from this herb.
Keywords: *M. malabathricum*, calcium oxalate, crystallization, lithiasis and total flavonoid content.

1. Introduction

The term lithiasis is referred to as the process of stone formation (Prywer & Torzewska, 2012). Stone usually affects different parts of the body such as calyx (upper, middle or lower part), renal pelvis, ureter (upper, middle or distal part) and urinary bladder. The formation of stone in the renal system is called urolithiasis. This disease affects a wide range of the population but up to now, despite advances in technological medical approach there is no satisfactory drug used in the treatment, prevention and reoccurrence of the disease (Butterweck & Khan, 2009). Up to 20% of the world population are suffering from urinary stone (Benramdane *et al*., 2008), Many parts of the world are suffering from urolithiasis, Malaysia is not excluded in terms of prevalence and incidence since it appears to show the same trend as in other developed countries (Sreenevasan, 1990). However despite its long history, economic drawback and mortality by this disease, there is no complete cure for this disease. Many plants claimed to have used for renal diseases were found to exhibit positive action (Sekkoum *et al*., 2010). The majority of stones are composed of calcium oxalate (Prywer & Torzewska, 2012).

Herbal medicinal plants have been used for the treatment of diseases since ancient time. In addition, it serves as important part of primary health care. Medicinal plants serve as important source of drug development and are also used traditionally to treat kidney stone.

It was also reported that *citrus aurantifolia, Centella asiatica L, Vernonia conferta* Benth*, Vernonia guineensis* Benth*, Brassica oleracea* L., *Canarium schweinfurthii* Engl, *Garcinia kola* Heckel, *Jatropha curcas* L., *Ricinodendron heudelotii (*Baill.) *Pierre ex Pax, Guarea cedrata* (A. Chev.*) Pellegr., Albizia adianthifolia* (Schum.) W.F. Wight*, Coula edulis Baill./Ebwelle, Ongokea gore (*Hua) Pierre*, Eleusine indica* (L.) Gaertn*, Oryza sativa* L., *Zea mays* L.*, Hallea ciliata* (Aubr. & Pellegr.) *Leroy, Fagara macrophylla* (Oliv.) Engl. syn, *Zanthoxyllum macrophyllum* Oliv*, Baillonella toxisperma* Pierre, *Cola lateritia* K. Schum and *Erismadelphus exsul Mildbr. Var. platyplyllus* were used by the people of Littoral Region, Cameroon as medicinal recipes in the treatment of urinary lithiasis (Noumi and Ebwelle 2011). However, there is no satisfactory drug to be use in clinical therapy, especially for the prevention and recurrence of stones (Butterweck & Khan, 2009). Since many medicinal plants were reported to have been used traditionally for the treatment of lithiasis, *M. malabathricum* was reported to have to have effect on renal disease in traditional medicine (Joffry *et al*., 2012). In line with this and also due to the reach chemical content of *M. malabathricum* the plant could be an important source in the management of lithiasis. Additionally, despite several studies carried out to assess the pharmacological activities of *M. malabathricum* there is no study conducted to find out whether the plant has inhibitory action on the growth of calcium oxalate crystals.

This study aimed to determine the inhibitory effects of calcium oxalate crystals growth by leaf, flower, fruit and stem of *M. malabathricum*. And to determine the relationship between total flavonoid content and anti-crystallization activity of different extracts.

2. Materials and methods

2.1 Chemical and Reagents

The chemicals, biochemicals and solvents used in this study were of analytical grade. These include: chloroform, ethanol, ethyl acetate, methanol sodium carbonate solution, methanol, aluminium chloride, sodium chloride, trisodium citrate dihydrate, disodium oxalate and silica gel 0.060-0.20mm all obtained from Merck, Germany. However, tris-hydroxymethylmethalamine (Tris) was obtained from Fisher Scientific UK. Calcium chloride dihydrate was obtained from Duchefa, Netherland.

2.2 Plant materials

M. malabathricum samples consisting of leaf, flower, fruit and stem were collected between April and August 2014 from the health forest Terengganu, Malaysia. The samples were identified by Science officer; Noorhaslinda Haron and deposited with the specimen vouchers 00244 in the Herbarium Unit, Department of Agriculture and Biotechnology, Faculty of Bioresources and Food Industry, Universiti Sultan Zainal Abidin (UniSZA).

2.3 Morphological Examination

Initially, the samples were checked by visual examination for basic macroscopic features such as length, shape, colour and odour.

2.4 Preparation of Extracts

The plant samples consisting of different parts (flower, leaf, fruit, and stem) were washed with tap water, then rinsed with distilled water and dried at 40°C. The samples were ground into powder form, and each powdered sample was weighed 600 g and soaked in methanol at the ratio of 1:10 (w/v) for 72 h. The extracts were decanted and filtered through Whatman filter paper no. 1. The filtrate was then concentrated in a rotary evaporator at low temperature 40°C and reduced pressure. The extracts were dried at 40°C and kept in a freezer at 4°C for further use.

A dried methanol extract (20 g) of *M. malabathricum* leaf was mixed with equal amount of silica gel and left to dry. The dried mixture was submitted to the column chromatography packed with silica gel earlier. The series of organic solvents with increasing polarity have been used starting with ethyl acetate (100%), followed by ethyl acetate : methanol at different ratios of (9 : 1), (8 : 2), (7 : 3) (6 : 4) and ending by 100% methanol. The fractions were collected in test tubes and combined based on the colour and similar profile on Thin Layer Chromatography. Eleven fractions were collected and coded as F1 to F11 respectively. The fractions were evaporated in a rotatory evaporator under reduced pressure at low temperature 35°C. The fractions were dried at 35°C in an oven and kept in a freezer at 4°C for further use.

The extracts of different part of *M. malabathricum* (leaf, flower, fruit and stem), sodium citrate and fractions of leaf (F1-F11) were prepared at 100 µg/mL using sodium chloride solution (0.15 M). Additionally, calcium chloride and sodium oxalate solutions at 40 mM and 4 mM respectively were also prepared using sodium chloride solution (0.15 M).

2.5 Total Flavonoid Content

The flavonoid content of different fractions of leaf of *M. malabathricum* obtained from column chromatography was determined according to the method of Moreno *et al.* (2000), with slight modifications. An aliquot of the extract measuring 0.25 mL in methanol at the concentration of 200 µg/mL was mixed with solution containing 50 µL of 10% aluminium chloride, 50 µL of 1 M aqueous potassium acetate and 2.15 mL of 95% ethanol. The mixtures were incubated at room temperature for 40 min and then the absorbance was recorded spectrophotometrically at 415 nm. The total flavonoid was calculated using quercetin as a standard and expressed as mg/g from the calibration curve**.**

2.6 Statistical Analysis

For total flavonoid content the experiments were carried out in triplicate and expressed as means ± SD. The data for the inhibition of calcium oxalate crystal growth and aggregation were expressed as mean ± standard error of mean (SEM). The means were compared by one-way ANOVA, and the values were considered to be significantly different at $P<0.05$. Spearman Correlation was used to estimate the interrelationship between TFC and antilithiasis activity of different fractions of *M. malabathricum* leaf.

2.7 Study of Inhibition of Calcium Oxalate Crystal Growth

Study of calcium oxalate crystallization in the absence of inhibitor has been carried out using two separate solutions of calcium chloride (40 mM) and sodium oxalate (4 mM). These solutions were prepared using sodium chloride solution (0.15 M) buffered with Tris 0.05 M at pH 6.8. A volume of 80 µL of 40 mM calcium chloride

was transferred into the well, and then to this, 80 µL of 4 mM sodium oxalate was added. The mixture was shaken to prevent sedimentation. The measurement of absorbance was immediately taken at 620 nm using Infinite M200 PRO TECAN and is continued for a period of 10 min. The solution of calcium chloride of 40 mM was used as blank and for each experiment,six replicates were taken. The data was used as the uncontrolled growth of the stone nucleus for the comparison of growth in the presence of the standard drugs and plant extract. The study was then carried out to assess the effect of plant extract and standard drug against stone nucleus formation. Plant extract and standard drug (sodium citrate) were used as inhibitors

Eighty µL of calcium chloride (40 mM) solution, prepared using sodium chloride solution (0.15 M) was transferred into the well (96 plate) and to this, 80 µL inhibitor (100 µg/mL) was added and also 80 µL of sodium oxalate (4 mM) was added to the mixture in wells, buffered with Tris 0.05 M at pH 6.8. The measurement of absorbance for the change in turbidity of the solution was immediately taken at 620 nm using Infinite M 200 PRO TECAN and continued for a period of 10 min with the mixture shaken to prevent sedimentation. The solution of calcium chloride (40 mM) and the inhibitor was used as blank. Each experiment was replicated six times and absorbance was recorded. The data was used as controlled growth of the stone nucleus. The data for both studies were compared, and the percentage inhibition of stone nucleus formation (crystallization) was calculated using the graphical method, using the equation below.

$$\% \text{ Inhibition} = \left\{1 - \left(\frac{A}{B}\right)\right\} \times 100$$

A = Turbidimetric slop in the presence of inhibitor.
B = Turbidimetric slop in the absence of inhibitor.
$CaCl_2 + Na_2C_2O_4 \rightarrow CaC_2O_4 + 2NaCl$

2.8 Aggregation Study on Calcium Oxalate Crystals

The study was carried out according to the method of Galani and Panchal (2014) with minor modifications. For the formation of calcium oxalate crystals, solution of calcium chloride and sodium oxalate at 50 mM were mixed and equilibrated at 60°C in a water bath for 1 h and cooled at 37°C for 24 h. The calcium oxalate crystals were collected and evaporated at 37°C. The calcium oxalate was weighed and prepared with sodium chloride at 1000 µg/mL and buffered with Tris 0.05 M (pH 6.8). The absorbance was taken at 620 nm for both in the presence and absence of inhibitor (100 µg/mL).
The percentage inhibition was calculated using the following formula:

$$\% \text{ Inhibition} = \left\{1 - \left(\frac{A}{B}\right)\right\} \times 100$$

A =Turbidimetric slope in the presence of inhibitor
B =Turbidimetric slope in the absence of inhibitor

2.9 Microscopic Study of Calcium Oxalate Crystals

The calcium oxalate crystals growth inhibition study of *M. malabathricum* was also carried out with the aid of microscopic examination.

2.10 Preparation of Slide in the Presence of Inhibitor

Small amount of calcium oxalate solution (1 mg/mL) buffered with Tris 0.05 M (pH 6.8) was place on to the slide and observed under light microscope.

2.11 Preparation of Slide in the Absence of Inhibitor

Small amount of solution containing equal amount calcium oxalate crystal (1 mg/mL) buffered with Tris 0.05 M (pH 6.8) and 100 ug/mL of either sodium citrate or extract of *M. malabathricum* was place on to the slide and observed under light microscope.

2.12 Microscopic Examination

The photographs of calcium oxalate crystals formed in the presence and absence of inhibitors, were observed using a light microscope (Olympus Microscope, BX43F-CCD), equipped with digital camera (Olympus DP21) and connected to a computer. The photographs were taken at times ten (10×) magnification objective lens.

3. RESULTS AND DISCUSSION

3.1 Morphology of M. malabathricum (Physical examination)

The variety of *M. malabathricum* used in this study has flowers with dark purple magenta petals and yellow pistil. The plant grows up to 1.8 meter in height. The leaves are odourless, dark green, ovale with average length and width measuring 12.5 -16 cm and 2.9-4.5 cm respectively and Figure 1. (a) shows the pictures of a leaf. The

fruits of the plant are purple in colour and oval in shape and when burst reveal sticky dark purple mass with orange seeds. Stem is reddish brown in colour. These findings are similar to the finding of Joffry *et al.* (2012). The colour of dried powdered leaf and fruit are pale green and red respectively whereas the colour of powdered dried stem and flower is brown and purple respectively. The picture of flower and seed of *M. malabathricum* is shown in Figure 1. (b) whereas the pictures of a close ups of the plant are shown in Figure 1. (c).

(a) (b) (c)

Figure 1. (a) Leaf of *M. malabathricum*, (b) Flower and fruit of *M. malabathricum,* (c) *M. malabathricum* plant

3.2 Total Flavonoid Content of Leaf Fractions

The TFC of leaf fractions obtained from column chromatography are presented in Table 1. and the result is expressed in terms of quercetin equivalent from the calibration curve equation: $y = 0.0046x + 0.0885$, $R^2 = 0.9941$. All fractions were found to contain flavonoid. F1 to F5 are less polar fractions (100% ethyl acetate), and they exhibited high flavonoid content. The F6-F9 are the mixtures of less polar and polar solvents (mixture of ethyl acetate and methanol) whereas F10 and F11 contain polar solvent (100% methanol) and these fractions showed low flavonoids content. The fraction with the highest flavonoid content was F1 with average TFC of 156.63 mg of QE per gram of extract. One-way ANOVA showed that, the flavonoid content is significantly different ($p < 0.05$) among fractions of *M. malabathricum* leaf. The post hoc test was carried out using Scheffe method.

Table 1. Total flavonoid content of fractions of *M. malabathricum* leaf extract

Fractions	TFC (mg QE/ g of extract)
F1	150.63 ± 16.89^{e}
F2	108.24 ± 12.03^{cde}
F3	122.55 ± 12.23^{de}
F4	120.38 ± 28.74^{de}
F5	79.98 ± 4.43^{bcd}
F6	58.61 ± 4.53^{abc}
F7	42.48 ± 6.80^{ab}
F8	70.74 ± 4.36^{abcd}
F9	28.71 ± 1.37^{ab}
F10	15.67 ± 5.76^{a}
F11	57.70 ± 31.64^{abc}

Data are expressed as mean ± SD. Data with different lower case letters on each part of the plant are significantly different ($p < 0.05$). The mean flavonoid content with identical alphabets show no significant difference ($p < 0.05$)

3.3 Crystallization of Calcium Oxalate

Crystallization study was carried out in the presence and absence of inhibitors. The study showed that, the extracts of different parts of M. *malabathricum* at 100 µg/mL inhibited calcium oxalate crystals formation. The leaf exhibited highest percentage of inhibition (90.63%) for calcium oxalate crystal formation and is statistically similar to that of positive control of sodium citrate (Table 2.), whereas stem showed lowest percentage of inhibition (63.02%) of calcium oxalate formation. However, the rate of crystallization of calcium oxalate crystals is statistically significant ($p < 0.005$) between different parts of *M. malabathricum*. Additionally, the effect of this plant on calcium oxalate crystallization may be associated with its high flavonoid content.

Table 2. Percentage inhibition of calcium oxalate crystallization of different parts of *M. malabathricum*

Sample	Percentage of inhibition (%)
Negative control	0
Positive control	95.31 ± 4.04^{b}
Leaf	90.63 ± 3.86^{b}
Flower	84.38 ± 2.05^{ab}
Fruit	66.40 ± 6.35^{a}
Stem	63.02 ± 6.62^{a}

Data are reported as the mean ± SEM of six replicates. The values with different letters are significantly different ($p < 0.05$) compared to the remaining groups

As a result of promising effects of methanol extract of leaf of *M. malabathricum*, further fractionation of the leaf extract was carried out. Eleven fractions of leaves were obtained from column chromatography, and each fraction was tested against calcium oxalate crystallization. F1 and F9 exhibited highest and lowest rate of inhibition of calcium oxalate crystallization respectively (Table 3.). It was found that most of the extracts inhibited the calcium oxalate crystallization not later than 10 min because there is no significant increase in the change of turbidity after 10 min. However, F1, F2 and sodium citrate inhibit crystallization of calcium oxalate crystal at 1 min. Consequently, the percentage of inhibition of nucleation of calcium oxalate crystals is statistically significant ($p < 0.005$) between different fractions. The Spearman correlation shows that, there is excellent significant correlation between TFC and percentage of inhibition of calcium oxalate crystallization with $r = 0.812$, $p = 0.002$. The finding of this study revealed that, there is an association between the TFC and antilithiasis activity in *in vitro* turbidimetry study of calcium oxalate. For this reason, it can be suggested that flavonoid compounds played important role in the inhibition of calcium oxalate crystals formation, and this is in line with the finding of Zhari *et al*. (1999). *M. mabathricum* is reported to have high flavonoid, and several flavonoids compounds such as kampferol, quercetin, quercitrin were isolated from this plant (Susanti *et al*., 2008). The results of its antilithiasis activity may be due to any of these compounds.

Table 3. Percentage of inhibition of calcium oxalate crystallization for different fractions of *M. malabathricum*

Fractions	Percentage of Inhibition (%)
Sodium Citrate	91.44 ± 5.73^{b}
F1	88.07 ± 11.46^{ab}
F2	54.51 ± 10.72^{ab}
F3	59.46 ± 5.46^{ab}
F4	59.01 ± 12.04^{ab}
F5	53.60 ± 8.31^{ab}
F6	53.83 ± 11.29^{ab}
F7	49.11 ± 12.42^{ab}
F8	63.75 ± 9.90^{ab}
F9	19.15 ± 13.72^{a}
F10	30.41 ± 15.95^{ab}
F11	58.11 ± 8.42^{ab}

Data are reported as the mean ± SEM of six replicates. The values with different letters are significantly different ($p < 0.05$) compared to the remaining groups

3.4 Aggregation of Calcium Oxalate

The aggregation of calcium oxalate was tested with fraction (F1) that had best activity on inhibition of calcium oxalate crystallization. The F1 fraction which had better activity inhibited the aggregation of already formed calcium oxalate crystals by 66.88%, whereas positive control (sodium citrate is used as positive control) inhibited the aggregation of calcium oxalate crystals by 76.33%. In addition to calcium oxalate crystal growth inhibition, *M. malabathricum* had also inhibited crystal aggregation. Therefore, the combined effect on crystallization, growth and aggregation is a clear indication that, *M. malabathricum* has antilithiasis activity *in vitro*.

3.5 Microscopic Examination

The microscopic examination of calcium oxalate crystals supported the turbidimetric assay. It has been shown in Figure 2. that there is high calcium oxalate crystals formation in the absence of sodium citrate. However, the photographs of Figure 3. showed very low formation of calcium oxalate in the presence of sodium citrate positive control and plant extracts. Both F1 fraction and sodium citrate inhibited crystallization and aggregation of calcium oxalate.

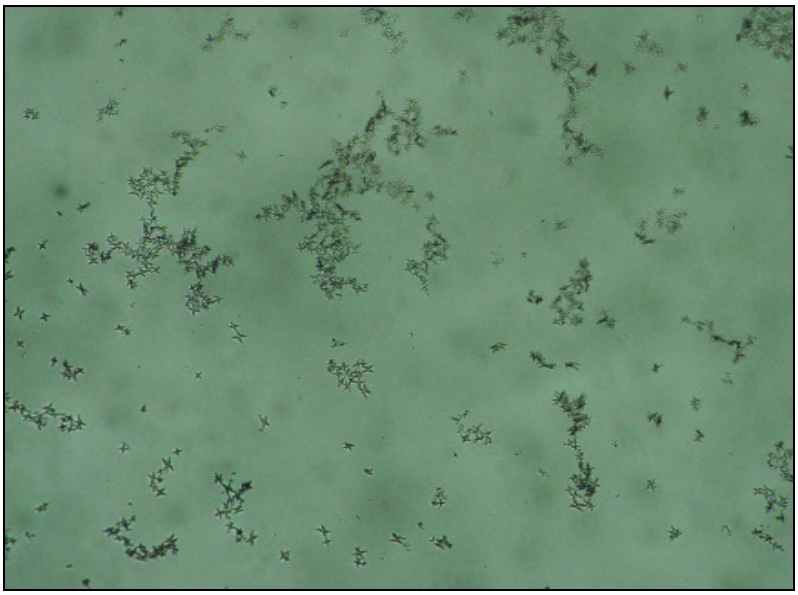
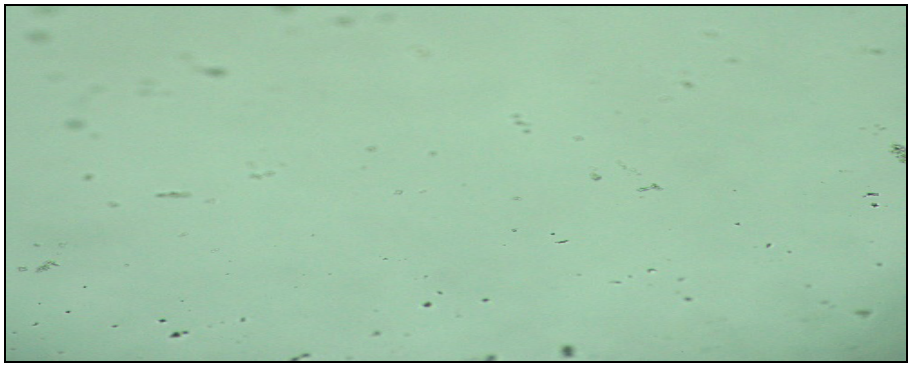
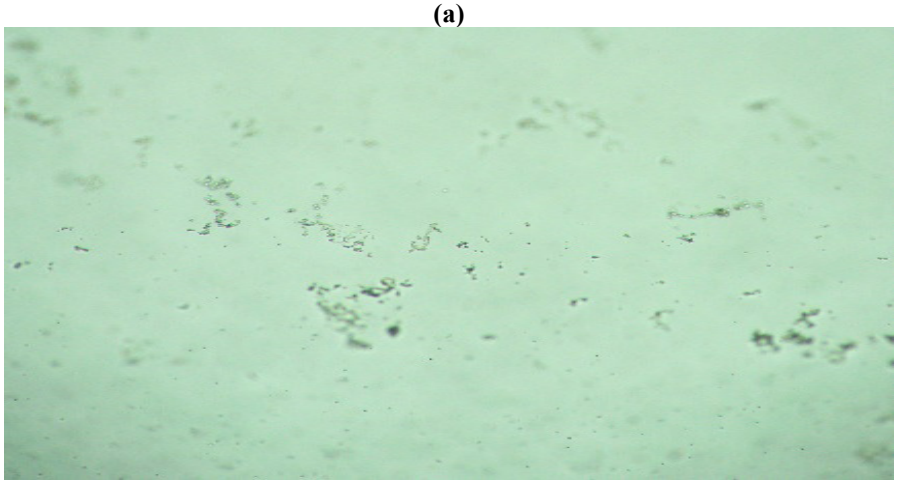


Figure 2. Photographs of calcium oxalate crystals in absence of sodium citrate (10×)



(a)



(b)

Figure 3. Photograph of calcium oxalate crystals in presence of (a) positive control of sodium citrate and (b) extract of *M. malabathricum* at 100 ug/mL (10×)

Both extracts of different parts of *M. malabathricum* and fractions of the leaf of *M. malabathricum* significantly inhibited the growth of calcium oxalate crystals by inhibition of crystallization and aggregation process. Therefore, the effect of *M. malabathricum* on calcium oxalate is similar to that of *Centratherum antihelminticum* seeds as it inhibited crystallization and aggregation of calcium *in vitro* (Galani & Panchal, 2014). According to the finding of this study, phenolic compounds especially flavonoids play a vital role in antilithiasis activity as shown by excellent correlation. Moreover, the parts of *M. malabathricum* with high total phenolic content and antioxidant potential were found to have high antilithiasis activity. In addition, the

antioxidant activity of *M. malabathricum* (Danladi *et al*., 2015) may partly explain its mechanism of antilithiasis activity.

4. CONCLUSION

This study shows that leaf of *M. malabathricum* inhibit all three step (crystallization, growth and aggregation) of calcium oxalate dihydrate crystals formation in vitro. Furthermore, the flavonoid compounds were founds to play an important role in preventing the crystallization and aggregation of calcium oxalate dihydrate crystals. The kidney stone formation involved three steps; crystallization, growth and aggregation. Therefore, *M. malabathricum* is suggested to be a potent antilithiasis agent as a result of its significant inhibitory effect against calcium oxalate crystals, which is the major cause of renal lithiasis. There is a need to carry out *in vivo* study, because, the exact mechanism of the initiation of the calcium oxalate stone formation is not completely understood. Additionally, not only excess calcium and oxalate crystals are contributing to the formation stone but other foreign body also played a role. It is also important to isolate the active chemical compound(s) responsible for this important therapeutic effect and to determine it is mechanism of action.

Reference

Benramdane, L., Bouatia, M., Idrissi, M. O. B., & Draoui, M. (2008). Infrared analysis of urinary stones, using a single reflection accessory and a KBr pellet transmission. *Spectroscopy Letters*, 41(**2**),72-80.

Butterweck, V., & Khan, S. R. (2009). Herbal medicines in the management of urolithiasis: Alternative or complementary?. *Planta Medica*, *75*(**10**), 1095–1103.

Danladi, S., Wan-Azemin, A., Najib, Y. S., Suryati, M. K., Rao, M. U. S., Mahsufi, S. M. & Dharmaraj, S. Phytochemical screening, total phenolic and total flavonoid content, and antioxidant activity of different parts of Melastoma malabathricum. Jurnal Teknologi. 2015, 77(2), 63-68.

Galani, V. J., & Panchal, R. R. (2014). *In vitro* evaluation of centratherum anthelminticum seeds for antinephrolithiatic activity. *Homeopathy & Ayurvedic Medicine*, *3*(**1**), 3–6.

Joffry, S. M., Yob, N. J., Rofiee, M. S., Affandi, M. R. M., Suhaili, Z., Othman, F., & Zakaria, Z. A. (2012). *Melastoma malabathricum* (L.) smith ethnomedicinal uses, chemical constituents, and pharmacological properties: A review. *Evidence-Based Complementary and Alternative Medicine*, 2012, 5-20.

Moreno, M. I. N., Isla, M. I., Sampietro, A. R., & Vattuone, M. A. (2000). Comparison of the free radical-scavenging activity of propolis from several regions of Argentina. *Journal of Ethnopharmacology*, *71*(**1**), 109–114.

Noumi E, Ebwelle E. S., (2011). Potentiality of Medicinal Plants in Treating Urinary Lithiasis in Littoral Region , Cameroon, *1*(3):74-87.

Prywer, J., & Torzewska, A. (2012). Effect of curcumin against *Proteus mirabilis* during crystallization of struvite from artificial urine. *Evidence-Based Complementary and Alternative Medicine*, *2012*.

Sekkoum, K., Cheriti, A., Taleb, S., Belboukhari, N., & Djellouli, H. M. (2010). Inhibition effect of some algerian sahara medicinal plants on calcium oxalate crystallization. *Asian Journal of Chemistry*, *22*(**4**), 2891–2897.

Sreenevasan, G. (1990). Urinary stones in Malaysia pits incidence and management. *Medical Journal of Malaysia*, *45*(**2**), 92–112.

Susanti, D., Sirat, H. M., Ahmad, F., & Ali, R. M. (2008). Bioactive constituents from the leaves of *Melastoma malabathricum* L. *Jurnal Ilmiah Farmasi*, *5*(**1**), 1–8.

Zhari, I., Sahabudin, R., Ismail, N., Ahmad, M., Sirajinisa, H., Raihan, S., & Temumen, A. (1999). Plant flavonoids as inhibitors of calcium oxalate urolithiasis. *Malaysian Journal of Science*, *18*, 13–15.