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Anti-Urolithiatic Terpenoid Compound from *Plantago major* Linn. (Ekor Anjing)

Aktiviti Anti-Urolitiatik Sebatian "Terpenoid" daripada *Plantago major* Linn. (Ekor Anjing)

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ABSTRACT

The aim of this study was to determine the inhibition effects of the terpenoid of Plantago major *on calcium oxalate crystals* in vitro *and to compare the effects of* Plantago major *with clinically used drugs like zyloric and potassium citrate for the treatment of urinary stone. Modified Schneider slide gel method was used for in vitro study and crystals formed were measured by Image Analyser System (Leica) after 24 h of treatment. The active compound in the methanol extract of* Plantago major *was isolated by bioassay - guided fractionation & isolation method. Dimethylsulphoxide (DMSO) was used as the negative control and zyloric and potassium citrate were used as positive controls. The results showed that crude methanol extract of* Plantago major *contained the active compound terpenoid. Terpenoid, zyloric and potassium citrate at concentrations in the range of (100 µg/mL - 250 µg/mL) significantly inhibited the area of crystal formation in comparison to the negative control after 24 h ($p<0.001$). The Zyloric and terpenoid of* Plantago major *in the concentrations of (100 µg/mL-250 µg/mL) inhibited the sizes of crystals significantly ($p<0.05$). Potassium citrate was more effective, than terpenoid of* Plantago major *in inhibiting the size of crystals at two concentrations i.e. 100 µg/mL and 150 µg/mL respectively ($p<0.05$). However the IC_{50} values for terpenoid of* Plantago major*, potassium citrate and zyloric were 250 µg/mL, 300 µg/mL and 550 µg/mL, respectively. The inhibition effect of the terpenoid of* Plantago major *extract on crystal size was much better than Zyloric and potassium citrate.*

Keywords: Calcium oxalate crystals; inhibition effects; Plantago Major*; terpenoid*

ABSTRAK

Kajian ini bertujuan untuk menentukan kesan perencatan sebatian terpenoid daripada ekstrak metanol Plantago major *terhadap kristal kalsium oksalat* in vitro *dan seterusnya membandingkan kesan* Plantago major *dengan dadah Zyloric and potassium citrate. Pada masa kini, kedua-dua dadah ini digunakan untuk merawat batu buah pinggang. Modifikasi kaedah Schneider slaid bergel telah digunakan untuk kajian* in vitro*. Selepas 24 jam, pembentukan hablur telah diukur menggunakan Sistem Analisis Imej (Leica). Sebatian aktif dalam ekstrak metanol* Plantago major *telah diisolasikan menggunakan kaedah Bioassay – fraksinasi terarah dan isolasi. Dimethilsulfhoksid (DMSO) telah digunakan sebagai kawalan negatif manakala Zyloric dan kalium sitrat pula telah digunakan sebagai kawalan positif. Keputusan kajian menunjukkan bahawa ekstrak* Plantago major *yang mengandungi compound terpenoid, zilorik dan kalium sitrat pada julat kepekatan 100 mg/mL-250 mg/mL berupaya secara statistik merencat pembentukkan kristal berbanding kawalan negatif selepas 24 jam ($p<0.001$). Perencatan perkembangan saiz kristal turut dikesan pada Terpenoid daripada* Plantago major *berbanding Zyloric pada kesemua kepekatan kajian ($p<0.05$). Kalium sitrat telah didapati lebih berkesan daripada* Plantago major *dalam merencat pembentukan hablur pada dua kepakatan iaitu 100 mg/mL dan 150 mg/mL ($p<0.05$). Walaupun begitu nilai IC_{50} untuk terpenoid, kalsium sitrat dan zyloric berada pada 250 mg/mL, 300 mg/mL dan 550 mg/mL. Kesan perencatan sebatian terpenoid dalam ekstrak* Plantago major *didapati lebih baik berbanding dengan zilorik dan kalsium sitrat.*

Kata kunci: Kesan inhibitori; hablur kalsium oksalat; Plantago major*; sebatian terpenoid*

INTRODUCTION

Urolithiasis is a condition of formation of calculus in the urinary system, i.e. in the kidney, ureter, urinary bladder or in the urethra (Shakarriz & Marshal 2001). Generally there are five different types of stones of which calcium oxalate is the most common (80%), struvite stone (10%), uric acid stone (9%) and (1%) is due to cystine and ammonium urate (Coe et al. 2005).

There are two types of calcium oxalate crystals i.e. monohydrate type (in the form of dump bell or oval in shape) and the dihydrate type (in the form of double pyramid) (Kannabiran & Selva 1997).

The cause is multifactorial including diet, genetic and environment (Anderson 1979). Many treatments have been tried for the treatment of urolithiasis in Malaysia and other parts of the World. It recurs back within five years (50%)

and there is not one standard treatment that can prevent the recurrences (Burkhill 1994).

Plantago major Linn. belonging to the family Plantaginaceae is a perennial herb which was found wild throughout the whole of Europe and temperate Asia (Burkill 1966). Every part of the plant has been used in many traditional medicines to treat cough, diarrhoea, dysentery and urinary tract calculus (Burkill 1966; Mabey 1988; Muhamad et al. 1994). Zyloric (Allopurinol) is a uricosuric agent that has been clinically used for the follow up patients with stone as it reduced the production of uric acid in urine and in fact uric acid may be a nidus for calcium oxalate crystals (Ismail et al. 1984). Potassium citrate is a low molecular weight inhibitor for crystallization (Meyer & Smith 1975).

The present study is on the inhibiting effects of terpenoid of *Plantago major in vitro* and to compare the effects of this extract with the clinically used drugs like zyloric and potassium citrate.

EXPERIMENTAL DETAILS

The whole plant (stem, leaves and roots) was collected from Air Itam, Penang and was identified by the botanist from Forest Research Institute of Malaysia (FRIM) where a voucher specimen was deposited. The herbarium registered number in FRIM is ZAS -1073/ SEPT. 2004. The fresh sample was washed, cut into small pieces and dried at room temperature for a week. The sample was ground into fine powder and Soxhlet extracted with methanol. Different concentrations of the extract were prepared by diluting with DMSO (dimethylsulfoxide) 100 ppm, 150 ppm, 200 ppm, 250 ppm (1 ppm =1 µ/mL). One and half milliliter of one percent (1%) bactoagar was heated to liquefy and then smeared on the clean glass slides and each slide was divided into two equal areas whereby eight equal wells (spacing between wells was 0.5 cm × 1.25 cm) were made in the gel when the gel was about to solidify (Schneider et al. 1983). The slides prepared were divided into negative control (DMSO only) (n=10), positive controls (zyloric & potassium citrate) (each n=10), *Plantago major* extract (n=10). For the production of calcium oxalate crystals, 20 uL of 0.2 M calcium chloride and 20 uL of ammonium oxalate was introduced in the opposite wells and the samples to be studied were put in the two longitudinal wells. The area and size of the crystal were measured after 24 h by using Image Analyzer System KS 300, 3.0 Leica. The magnification was 320X.

IDENTIFICATION OF ACTIVE COMPOUND SOLVENT PARTITIONING

The separation method of crude extract of *Plantago major* based on the polarity of the components known as solvent partitioning. In this study, crude extract was divided into 5 other different extracts like hexane, chloroform, ethyl acetate and aqueous extracts.

About 700 mL of distilled water was poured into a beaker containing crude extract of *Plantago major*. The crude extract of *P. major* was dissolved in distilled water was then transferred into a separating funnel (700 mL). Hexane solution was then introduced into the separating funnel. The separating funnel was then stoppered and shaken for 5 min to allow the components in the solution to be extracted by hexane.

After hexane extraction was done, chloroform extraction was done followed by extraction of ethyl acetate. The steps in extraction of chloroform and ethyl acetate were the same as that for hexane extraction. Chloroform extract differs from hexane and ethyl acetate extracts as chloroform extract has a higher density compared to aqueous extract. Thus, chloroform extract was found below the aqueous layer.

At the end of the extraction process, the remaining aqueous extract was collected after obtaining the acetyl acetate extract. Each extract collected was dried using

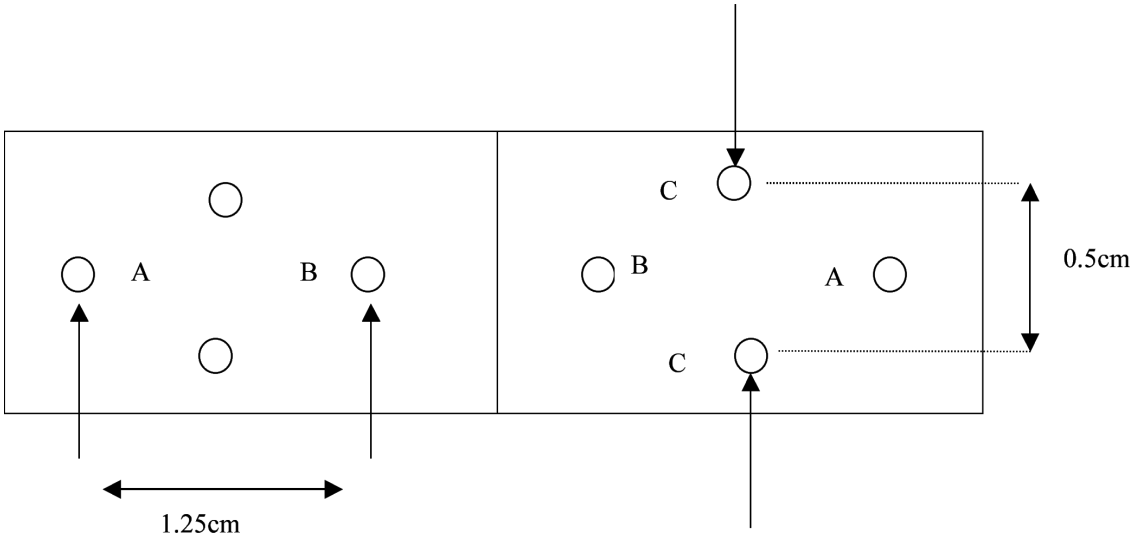

FIGURE 2. Modified Schneider slide gel method in making 2 areas of crystal formation at one slide

rotavapour at 45°C, except for aqueous extract which was dried at 50°C.

PROCESS BIOASSAY- GUIDED FRACTIONATION & ISOLATION OF ACTIVE COMPOUND

Bioassay –guided fractionation process was a step to test the activity of sample extract of *P. major*. Flow chart of bioassay-guided fractionation process and separation of the active compound is shown in Figure 2. The activity of hexane, chloroform, ethyl acetate and aqueous extracts obtained by solvent partitioning were tested and the one with the highest activity was selected for further separation. The first level activity test showed that the aqueous extract has the highest activity. Activity test was done by modified gel slide method of Schneider et al. (1983).

Aqueous extract was selected to undergo further separation in order to get the active compound in *P. major* that was actively inhibiting the formation of crystals in vitro. Chromatography column was used in the separation process of active compound. The initial separation used Diaion HP_2Oss column. A total of 7 fractions were obtained by Diaion HP_2Oss column. Activity test done showed that the 5th fraction (A5) was the most active fraction.

Fraction A5 being most active was selected for further separation in order to obtain a single active compound. Further separation was carried out by Chromatography column using ODS column (Chromatorex) and sulphadex LH20 were used. A total of 8 fractions were obtained using this column. The 4th fraction (A51V) was the most active fraction. Thin layer chromatography (TLC) was used to identify the active compound in A51V which can effectively inhibit the formation of crystals in vitro.

IDENTIFICATION OF ACTIVE COMPOUND IN *PLANTAGO MAJOR*

Thin Layer Chromatography (TLC) was used to identify the active compound in this study. TLC plate measuring (4×5 cm^2) was prepared. A spot was marked on the plate

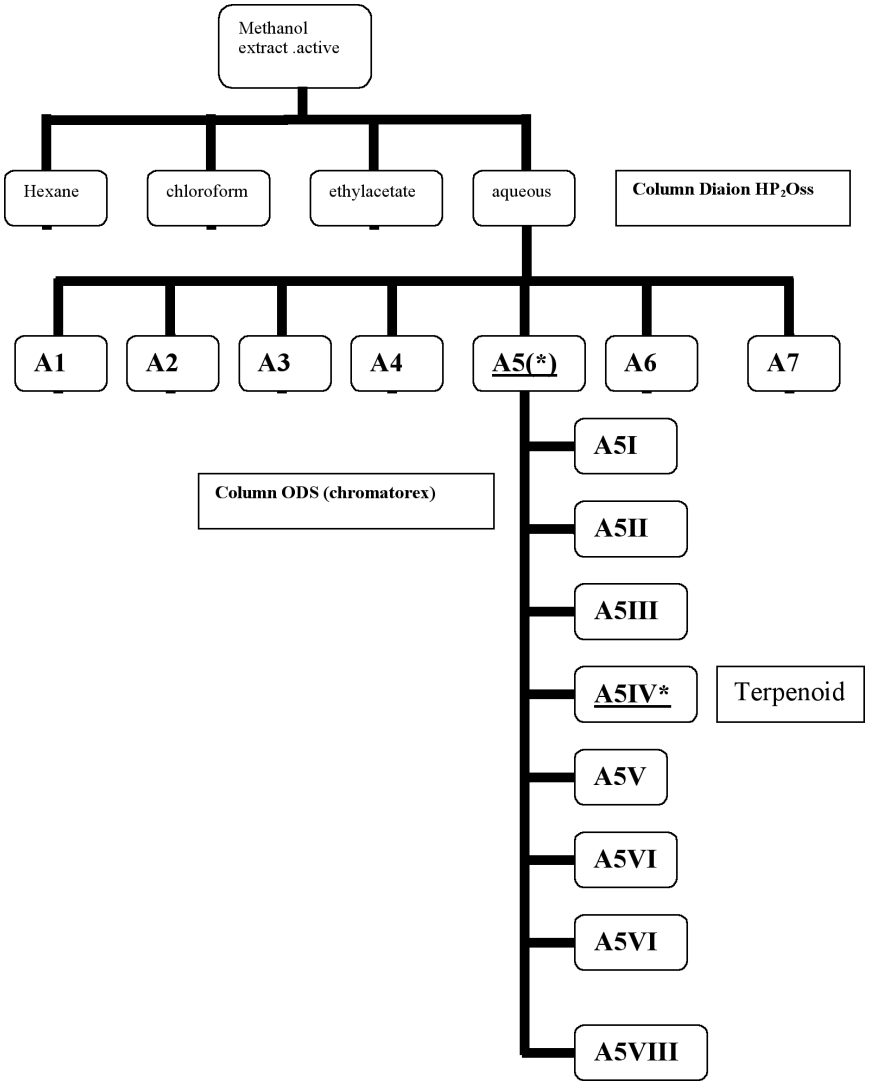

FIGURE 2. Bioassay-guided Isolation of Active compound in the methanol extract of *Plantago major*

about 0.5 cm from the bottom end of the plate. Capillary tube was used to transfer 3 drops of the sample to the drop that has been drawn. Then the plate was introduced into a beaker filled with solvent (chloroform : methanol : distilled water = 7:3:0.5) that serves as the moving phase. The beaker was covered and when the solvent has moved about 0.5 cm from the upper end of the plate, the plate was taken out and dried.

The TLC plate was observed under UV light and spots seen on the plates was identified. Next, the plate was sprayed with sulphuric acid 10% and warmed on hot plate. Colored patches were produced and the active compound present was identified based on the color of the patches (eg. Terpenoid- pink to purple/violet colour).

IC50 was determined by finding out the concentration of terpenoid, zyloric and potasium citrate which can reduce the size of calcium oxalate crystals to about half the size mean size of normal control (i.e DMSO) (Table 1).

Results and Discussion

The calcium oxalate crystals that have been produced in this *in vitro* study were similar to the crystals in the urine of patient with calcium oxalate crystals (Zhari et al. 1995). Most of the crystals measured in this study were calcium oxalate (dihydrate type) since 90% of monohydrate type were formed only after 48 h Dimethyl sulphoxide (DMSO) was used as control since DMSO was widely used as industrial solvent for both organic and inorganic chemicals (Whillhite & Datz 1984).

In this study methanol extract of *Plantago major* has been used since many of the active compounds in the plants were dissolved in it and can be extracted. Because the polarities of four different solvents like hexane, chloroform, ethyl acetate and aqueous are different, the active compound in them is also different (Fasihuddin & Hasmah 1993). The results of the *in vitro* inhibition effects of different concentrations of terpenoid of *Plantago major*, Zyloric and potassium citrate on the area of calcium oxalate crystal formation after 24 hours were shown in Table 2. This study showed that *Plantago major*, Zyloric and potassium citrate at concentrations of 100, 150, 200, 250 µg/mL significantly inhibited the area of crystals formation when compared to negative control after 24 h ($p<0.001$). Terpenoid of *Plantago major* was more effective in reducing the area of crystal formation compared to

TABLE 2. IC_{50} values of the terpenoid of *Plantago major*, Zyloric and potassium citrate on the size of calcium oxalate crystals after 24 hours *in vitro*

Sample	IC_{50}values (µg/mL).	Mean size crystal (μm^2)
DMSO (negative control)	-	147.68±3.5
Terpenoid of *Plantago major*	250	73.95±4.4
Zyloric (positive control).	550	72.3±2.95
Potasiium citrate (positive control)	300	74.9±5.1

Clearance = IC_{50}value is the inhibitory concentration of the tested compound which can reduce the mean size of calcium oxalate crystals to half the size or nearly half the size of that of control (i.e DMSO)

TABLE 2. Inhibition effects of terpenoid of *Plantago major*, Zyloric and potassium citrate at different concentrations on area of calcium oxalate crystal formation after 24 hours *in vitro*. * $p<0.001$ vs control, # $p<0.05$ vs extract of *Plantago major* (unpaired t-test)

Sample	n	Different concentrations in (µg/mL)	Area of crystal formation in mm^2 (mean±S.E.M).
Terpenoid of *Plantago major*	10	100	7.71±0.2 *
		150	7.05±0.2 *
		200	5.62±0.3 *
		250	5.32±0.3 *
Zyloric (positive control)	10	100	7.5±0.5 *
		150	6.7±0.3 *
		200	6.05±0.3 *
		250	5.6±0.3 *
Potassium citrate (positive control)	10	100	8.7±0.5 *
		150	7.7±0.3 *
		200	6.6±0.2 *#
		250	6.4±0.3 *#
DMSO (negative control)	10	100	11.93±1.63
		150	11.49±1.55
		200	11.59±1.39
		250	11.84±1.26

Clearance – S.E.M means standard error of mean

potassium citrate at 200 and 250 µg/mL ($p<0.05$), whereas there were no significant differences between the effects of Zyloric and *Plantago major*. The exact mechanism of action is not known but the active ingredient in *Plantago major* may have inhibited the growth or aggregation of the crystals or it may have dissolved the preformed crystal (Zhari et al. 1995).

Table 3 shows the inhibitory effects of terpenoid of *Plantago major*, zyloric and potassium citrate on the size of each crystal (dihydrate variety) as compared to control. The reduction in the size of calcium oxalate crystals by *Plantago major* was greater compared to zyloric at all concentrations tested ($p<0.05$). Potassium citrate was more effective than *Plantago major* in reducing the crystal size at the lower concentrations of 100 and 150 µg/mL. However, IC_{50} values of terpenoid of *Plantago major*, potassium citrate and zyloric were 250 µg/mL, 300 µg/mL and 550 µg/ml respectively for the reduction of crystal size (Table 1). The results indicated that terpenoid of *Plantago major* was much better than zyloric and potassium citrate in reducing the size of the crystals. Abramson (1992) has identified polysaccharides, fat, caffeine acid derivatives, flavonoids, glycosides, irinoid and terpenoids. In this study the active ingredient in *Plantago major* that has inhibition effects on the area and size of calcium oxalate crystals was terpenoid. Calcium oxalate crystals (dihydrate type) that has been inhibited in sizes by all four groups studied in this research is shown in Figure 3. Comparing the size of crystals, the most effective reduction on the size of crystal was terpenoid followed by potassium citrate and zyloric. Terpene is a one of the important secondary metabolites in plants. When terpene is modified chemically, it is called terpenoid. There are variety of terpene namely monoterpene, diterpene, sequisterpene and others. Plant terpenoids are used extensively for their aromatic qualities. They play a role in traditional herbal remedies and are under investigation for *antibacterial, antineoplastic*, and other *pharmaceutical* functions. Terpenoids contribute to the scent of *eucalyptus*. *Plantago major* has been found to contain iridoid glycosides of which aucubin is a major triterpenes (Ibrahim 2006).

Conclusion

In conclusion the significant inhibiting effect of the terpenoid of *Plantago major* on the area of calcium oxalate stone formation was observed at 100µg/mL onwards ($p<0.001$) with an IC_{50} value of the terpenoid being 250 µg/mL. Therefore the effect of terpenoid from methanol extract of *Plantago major* was more effective than zyloric and potassium citrate in inhibiting the sizes of calcium oxalate crystals *in vitro*. In conclusion the active compound in methanol extract of *Plantago major* that inhibited the area of formation and size of calcium oxalate crystals was compound terpenoid and effect of compound terpenoid was much better that zyloric and potassium citrate.

Further study the effect of compounds in *Plantago major* extract on urine of urolithiatic patients to see how effective it is on crystals of stone patient and also clinical trials are needed on the human beings with calcium oxalate urolithiasis. There may be a potential for using alternative medicine for patients with urolithiasis apart from surgery and western medicines which is practiced at present.

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TABLE 3. Inhibition effects of terpenoid of *Plantago major*, Zyloric and potasiium citrate at different concentrations on mean size of calcium oxalate crystals after 24 hour *in vitro*. *p<0.05 vs negative control, # p<0.05 vs *P*

Group	n	Different concentrations in µg/mL	Size of calcium oxalate crystals in μm^2 (Mean±SEM)
Terpenoid *of Plantago major*	50	100	126.9±5.3 *
		150	116.5±3.5 *
		200	94.6±1.9 *
		250	73.9±4.4 *
Zyloric (positive control)	50	100	142.2±5.2 *#
		150	138.5±4.3 *#
		200	137.5±3.7 *#
		250	132.8±5.8 *#
Potassium citrate (positive control)	50	100	102.8±2 *#
		150	92.8+2.6 *#
		200	89.3+3.5 *
		250	84.8+3.6 *
DMSO(negative control)	50	100	147.7±3.5
		150	147.4±3.9
		200	146.9±4.5
		250	146.9±3.5

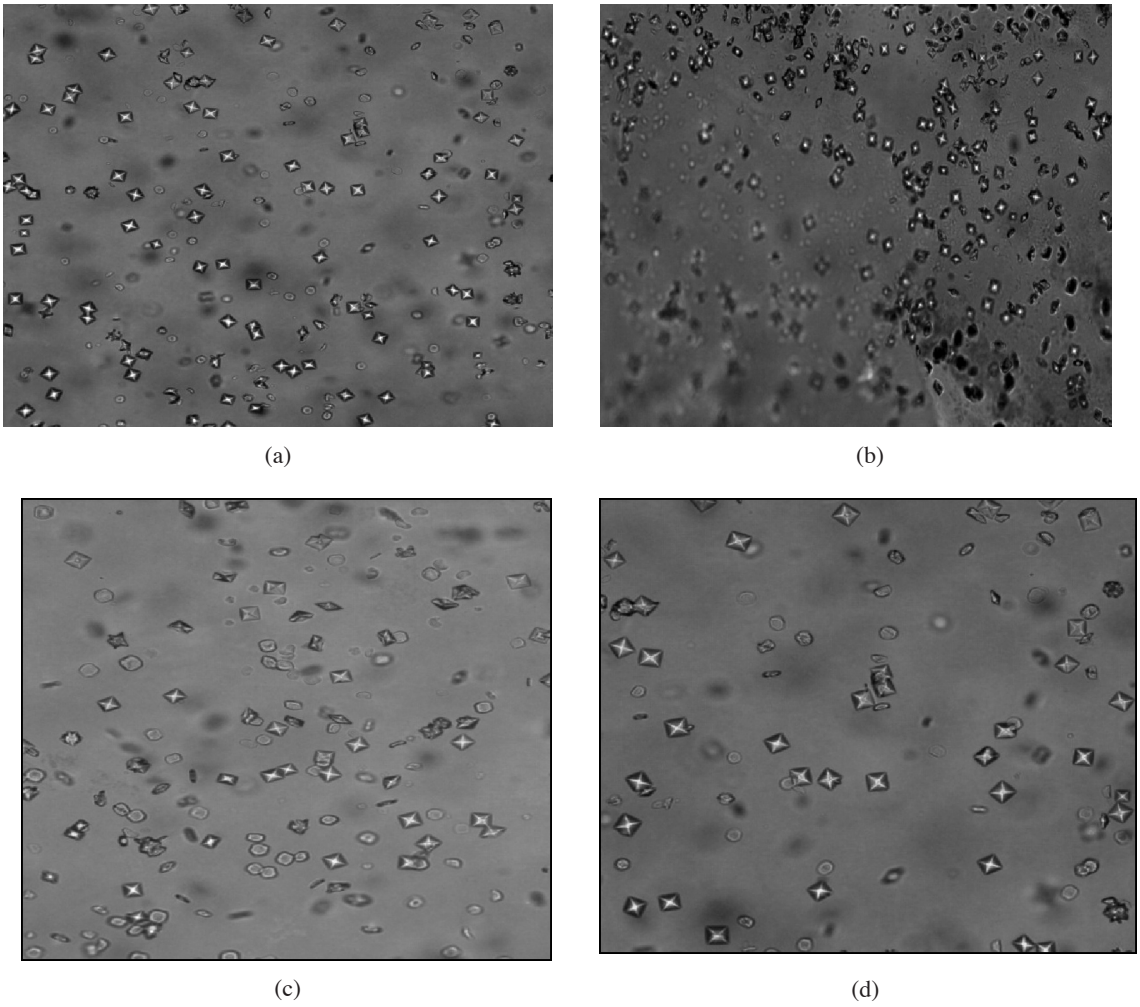

FIGURE 3. Photograph showing the inhibited calcium oxalate crystals' sizes (dihydrate variety) by compound Terpenoid , zyloric and potassium citrate vs control. The magnification of Image Analyser system is 320×. (a) Potassium citrate (positive control), (b) Terpenoid of Plantago major, (c) Zyloric (positive control) and (d) Control: Dimethylsulphoxide

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