DOI: 10.56511/JIPBS.2018.v5.i01.pp42-45

Journal of Innovations in Pharmaceutical and Biological Sciences (JIPBS)
ISSN: 2349-2759
Available online at www.jipbs.com

Research article

Antinephrolithiasis effect of ethanol extract of *Phaleria macrocarpa* (Scheff.) boerl in male wistar rat

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Key words: Calcium oxalate, Ethylene glycol, Nephrolithiasis, Flavonoids

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Abstract

Objective: The aim of this research was to determine *in vitro* and *in vivo* antinephrolithiasis activity. **Methods:** *In vitro* nephrolithiasis activity was performed by incubating calcium oxalate and ethanol extract of *Phaleria macrocarpa* fruit at 37°C for six hours. *In vivo* test was induced by administration of 0.75% ethylene glycol and 2% ammonium chloride for 10 days and continued to ethanol extract of *Phaleria macrocarpa* fruit administration for 10 days then determined calcium oxalate concentration. **Results:** The *in vitro* results showed that ethanol extract of *Phaleria macrocarpa* significantly dissolved calcium oxalate. The *in vivo* result showed that ethanol extract of *Phaleria macrocarpa* fruit decrease calcium oxalate concentration of kidney at dose of 200 and 400mg/kg BW were significantly different ($P< 0.05$) to negative control. **Conclusion:** Ethanol extract of *Phaleria macrocarpa* fruit has antinephrolithiasis activity on *in vitro* and *in vivo*.

Introduction

Renal stone is a common disease, occurring in 8% of the population. This disease is multifactorial and mainly considered related to environmental factors, especially western diet. Calcium stones are encountered in 80% of cases and contain calcium oxalate (72%), phosphate oxalate (14.7%) and often a mixture of the two [1]. The cause of the formation of urinary tract stones may occur because urine is saturated with salts that can form stones or because urine lacks inhibiting normal stone formation. About 80% of the stones consist of calcium, the rest contains several elements, including uric acid, cystine and struvite minerals [2].

Stage formation of calcium oxalate crystals in the body begins with high calcium levels bound to oxalate to form calcium oxalate crystals, then more and more calcium oxalate crystals. The saturation of substance forming stone in the urine such as cystine, xanthine, uric acid, calcium oxalate will facilitate the formation of stones. The formed crystals are retained in the kidneys [3].

Ethylene glycol is an odorless, colorless, liquid, sweet and toxic alcoholic derivative. Ethylene glycol in the body is converted to oxalate, which subsequently binds to calcium to form calcium oxalate and will accumulate in crystalline form particularly in the kidney region [4,5]. Ethylene glycol administration is a well-known model of nephrocalcinosis. Ethylene glycol metabolizes into glycolate, glyoxylate, and oxalate leading to calcium oxalate crystals in both urine and kidneys [6]. Rats receiving Ethylene glycol supplemented drinking water (0.75% v/v) develop hyperoxaluria and hypercalciuria one day after initiation [7].

The *Phaleria macrocarpa* is a tropical plant originating from the papua area which is often used in traditional medicine [8]. In *Phaleria macrocarpa* leaves, containing antihistamines, alkaloids, saponins and polyphenols (lignans); on the skin of the fruit there are alkaloids, saponins, and flavonoids; while in the fruit there are alkaloids, tannins, flavonoids, phenols, saponins, lignans, essential oils, and sterols.

The *Phaleria macrocarpa* fruit is known to have a high flavonoid content [9], in which flavonoids can work to reduce free radicals by preventing lipid peroxidation triggered by calcium oxalate crystals, the flavonoid breaks the calcium bonds that exist in kidney stones and binds some calcium.

Materials and methods

Materials

Ammonium oxalate, aquabidest, calcium chloride, nitric acid 65%, standard solution of calcium 1000 mg/mL, Ammonium chloride, Batugin Elixir® (Kimia Farma), demineralized water, ethylene glycol, ethanol extract of *Phaleria macrocarpa* fruit.

Methods

Plant collection

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The plant material used is the fruit of the fresh *Phaleria macrocarpa* obtained from the yard of JalanPermai house, Medan District Struggle Medan North Sumatra. Identification of plant material was done in "Herbarium Bogoriense", Center for Biological Research and Development-LIPI, Cibinong, Bogor

Preparation of ethanol extract of *Phaleria macrocarpa* fruit

The simplicia powder is inserted into a glass container, added with 96% ethanol, sealed, left for 5 days shielded from light while stirring frequently, scrutinized, squeezed, then washed with sufficient liquid to obtain juice. Transferred into a closed vessel, left in a cool place protected from light for 2 days, poured. The results obtained were concentrated by means of the rotary evaporator method used by Pharmacopeia Indonesia third edition [10].

Phytochemical screening of ethanol extract *Phaleria macrocarpa*

Phytochemical screening performed on *Phaleria macrocarpa* ethanol extract included examination of secondary metabolites of alkaloids, flavonoids, glycosides, tannins, saponins, triterpenoids, and steroids [19-20].

Preparation of sample

Preparation of calcium's calibration curve

A total of 5 mL of 1000 ppm calcium (the mother liquor) was added to a 100 mL volumetric flask and then added aquabidest right to mark boundaries, the obtained raw calcium 50 ug/mL. Each of 1 mL, 2 mL, 3 mL, 4 mL, and 5 mL pipetted out from calcium standard solution of 50 ug/mL in a 50 mL volumetric flask to obtain successive concentration of 1 ppm; 2 ppm; 3 ppm; 4 ppm and 5 ppm and measured by atomic absorption spectrophotometry at a wavelength of 422.7 nm. Then obtained a calibration curve of calcium.

Analysis of calcium value *in vitro*

The ethanol extract of *Phaleria macrocarpa* fruit is made in several concentrations, ie 0.25% (A); 0.5% (B); and 1% (C). Weighing each extract, included in a 100 ml quantity flask, was sufficiently volume with an demineralized water to the mark line, transferred into a 250 ml erlenmeyer, incorporated calcium oxalate and incubated at 37°C for 6 hours with shaking at one hour intervals. Each concentration is made in six repetitions [11].

Destruction of samples *in vitro*

An amount of 100 mL for samples was added by 10 mL nitric acid 65 % and heat on a hot plate until the extract solution become transpicuous.

Determination of calcium levels in sample

The absorbance of sample solution that has been prepared was measured using flame atomic absorption spectrophotometry at a wavelength 422.7 nm. Absorbance values obtained should be within the range of the calibration curve of calcium standard solution. Levels of calcium are calculated based on the regression equation of the calibration curve [12].

Selection of animals for *in vivo* studies

For the purpose of anticalculi studies, adult male Wistar albino rats weighing around 200 g were selected. The animals were acclimatized to standard laboratory conditions and maintained for 12 hours light and dark cycle. They were provided with regular rat chow and drinking water ad libitum. Our Institutional Animal Research Ethics Committees (AREC) Approval No: 359/KEPH-FMIPA/2017.

Animal groupings

Animals were divided into six groups, each with five rats. The group I act as normal group. Group II, act as calculi negative control where animals received 0.75% ethylene glycol with 2% ammonium chloride in drinking water for 10 days. Group III (positive control) animals received 0.75% ethylene glycol with 2% ammonium chloride in drinking water along with Batugin Elixir® drug from the first day untill the tenth day. Rats in group IV, V and VI were treated respectively with ethanol extract of *Phaleria macrocarpa* fruitdose 100, 200 and 400 mg/kg BW from day eleven till last day.

Destruction of samples *in vivo*

Each kidney obtained from the rats was dried in oven (100^{o}C) for 24 hours and then heated in 540^{o}C for eight hours and then diluted with nitric acid and demineralized water.

Determination of calcium levels in kidney

The absorbance of sample solution that has been prepared was measured using flame atomic absorption spectrophotometry at a wavelength of 422.7 nm for calcium. Absorbance values obtained should be within the range of the calibration curve of calcium standard solution. The calcium level will be calculated based on the regression equation of the calibration curve [14].

Statistical data analysis

Results were given as Mean ± SEM from five animals in each groups. Comparisons were made by ANOVA method with SPSS 21. P (probability value) < 0.05 was considered significant.

Results and discussion

Phytochemical screening result of ethanol extract *Phaleria macrocarpa*

The results of the phytochemical scheme show that ethanol extract *Phaleria macrocarpa* positively contains of flavonoids, alkaloids, saponins, tanins, glycosides and steroids/triterpenoid.

Calibration curve of calcium

Both of this calibration have the same range for the concentration which were measured on the concentration of 1 ppm, 2 ppm, 3 ppm, 4 ppm and 5 ppm. The correlation coefficient obtained from this metal can be accepted as the appropriate requirements for the correlation coefficient which should not smaller than 0.9995. Coefficient above suggested a linear relationship between the concentration of the metal and absorbance [13]. The calibration curve of calcium is shown in Figure 1.

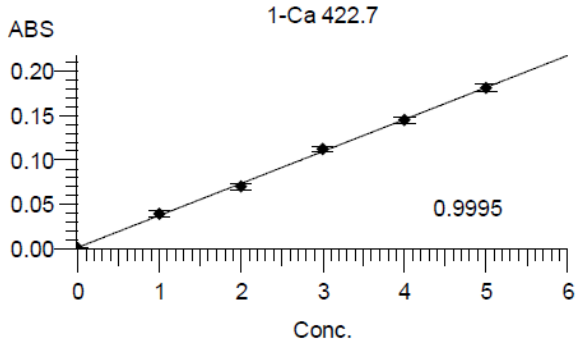


Figure 1. The calibration curve of calcium standard solution.

The Effect of calcium oxalate solubility in ethanol extract of *Phaleria macrocarpa in vitro* studies

The solubility testing of calcium oxalate by ethanol extract of *Phaleria macrocarpa* fruit was conducted with a view to determine the potential of early anticalculi effect by immersing 100 mg of calcium oxalate in 100 mL ethanol extract of *Phaleria macrocarpa* fruit concentration 0.25(A); 0.5(B) and 1% (C).

Table 1. Level of Calcium in Sample

Extract group	Early Ca's Level (µg/mL)	Ca's Level After Incubation (µg/mL)	Solubility Level (µg/mL)	% of Solubility
A	66.98	145.10	78.11	53.83
B	50.08	258.82	208.74	80.44
C	129.30	279.05	149.75	81.13

According to the table 1 can see that the level of calcium oxalate dissolved in A is equal to 78.11 µg / ml with a percentage of 53.83% solubility. Levels of calcium oxalate dissolved in B is equal to 208.74 µg / ml with a percentage of 80.44% solubility. Levels of calcium oxalate dissolved in C is equal to 149.75 µg / ml with a percentage of 81.13% solubility. Percent solubility of calcium oxalate in the C group was higher than A group. This is likely due to the levels of flavonoid in the C group was higher than A group [15].

The Effect of calcium oxalate solubility in ethanol extract of *Phaleria macrocarpa in vivo* studies

Metabolites of ethylene glycol such as glycolaldehyde, glycolate and oxalate can induce tissue damage. hyperoxaluria and the calcium oxalate calculi. In these studies chronic administration of 0.75% ethylene glycol and 2% ammonium chloride to male albino Wistar rats increased calcium level in the kidney (Group II/ Control Negative) [16].

The calcium level was significantly increased when compared to normal animals are shown in table 2.

The calcium levels were decreased while treated with ethanol extract of *Phaleria macrocarpa* fruit. The treatment with ethanol extract of *Phaleria macrocarpa* fruit dose 200 and 400 mg/kg BW significantly decreased the calcium levels compared to negative control ($P < 0.05$). These results indicated that ethanol extract of *Phaleria macrocarpa* fruit treatment shows improvement in renal function compared to the group II/ control negative.

Factor to dissolve calcium oxalate is flavonoids in ethanol extract of *Phaleria macrocarpa* fruit. The other research investigation also said that the effect of inhibiting calculi is from the antioxidant effect of the flavonoids. It seems that flavonoids in extract could play an antioxidant role against oxidative stress that is induced by the ethylene glycol [17-18].

Table 2. Calcium level in kidney

Group	Subject	Calcium level in kidney
(I)Normal	5	0.87 ± 0.03^{b}
(II) Negative control	5	4.40 ± 0.45^{a}
(III) Positive control	5	1.82 ± 0.22^{b}
(IV) Ethanol extract of *Phaleria macrocarpa* fruit dose 100 mg/kg BW	5	2.64 ± 0.27
(V) Ethanol extract of *Phaleria macrocarpa* fruit dose 200 mg/kg BW	5	2.35 ± 0.18^{b}
(VI) Ethanol extract of *Phaleria macrocarpa* fruit dose 400 mg/kg BW	5	1.99 ± 0.12^{b}

(Values are expressed as mean±SEM, n=5 a (group IV. V and VI vs group I). b: (group IV. V and VI vs group II)).

Conclusion

From the anticalculi test result, it can be concluded that ethanol extract of *Phaleria macrocarpa* fruit solution has the potential as anticalculi. From *in vitro* test result, it can be seen from the solubility percentage of calcium oxalate at 53.83%; 80.44% and 81.13% per six hours of treatment. From the *in vivo* test result, it can be seen from the decreased calcium level in the kidney on 0.75% ethylene glycol with 2% ammonium chloride induced nephrolithasison male wistar rat.

Acknowledgement

This research was supported by University of Sumatera Utara. Medan. Indonesia.

Conflict of interests

The authors declare that they have no conflict of interests.

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