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**Original article**

# Antiosteoporotic effect of sequential extracts and freeze-dried juice of *Cissus quadrangularis* L. in ovariectomized mice

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***Background:*** Osteoporosis becomes a major health problem in aging populations, and this disease increases the risk of bone fractures, leading to disability and mortality. *Cissus quadrangularis* L. (CQ) has been reported to have beneficial effects on bone metabolism; however, there has been no investigation to identify the active compounds responsible for this activity.

***Objective:*** Sequential extracts (hexane, dichloromethane, ethanol, and water) and freeze-dried CQ juice were investigated to determine their effects on bone metabolism in an ovariectomized (ovx) mouse model.

***Methods*:** Six-week-old ICR mice were divided into eight groups: sham-operated, ovx-control, estradiol ($E_2$)-treated and five CQ-treated ovx-mouse groups (n = 3). The CQ extracts were orally administered at a dose equivalent to 5g of crude powder/kg/day for 8 weeks. Bone mineral densities (BMD) of the femur and tibia, serum levels of osteocalcin (bone formation marker) and TRAP5b (bone resorption marker), and histomorphological change of lumbar spine were determined at the end of the experiment.

***Results*:** The BMD of the femur and tibia in the hexane-treated group were elevated to the same level as those of sham-operated group. This BMDs correlated with restoration of the trabecular bone of the lumbar spine, which was only observed in the hexane-treated group. These results were also supported by the lowest serum levels of osteocalcin and TRAP5b observed in this group, compared to the ovx-control and $E_2$-treated groups, representing a decrease in the bone turnover rate. Neither signs of abnormality nor pathological changes of internal organs were observed after the experiment.

***Conclusion*:** The hexane extract possessed antiosteoporotic activity in ovariectomized mice without any toxicity throughout the experiment. Therefore, the hexane extract is the most interesting for further bioassay-guided purification of pharmacologically active compounds.

***Keywords:*** Bone mineral density, medicinal plant, osteocalcin, osteoporosis, TRAP5b, Vitaceae

Osteoporosis is the result of an imbalance in the bone remodeling process in which the rate of bone resorption exceeds the rate of bone formation, and consequently, the bone mineral density (BMD) declines. This phenomenon increases the risk of osteoporotic fracture, especially in the vertebrae and hip. These fractures lead to morbidity and mortality, which is more pronounced in the elderly [1].

Bone turnover, or bone remodeling, is a process that occurs throughout life to maintain the macroscopic shape and integrity of bone, to repair microdamage, and to maintain calcium and phosphate homeostasis [2]. Bone turnover involves two processes, bone resorption and bone formation, and a change in one process will affect the other. Bone density is dependent

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on the net rate of both processes [3]. As a result of this, in this study, tartrate-resistant acid phosphatase isoform 5b (TRAP5b), a specific enzyme marker of bone resorption, and osteocalcin, a specific protein marker of bone formation, were used to determine the overall image of bone turnover [4, 5].

*Cissus quadrangularis* L. (CQ), a succulent perennial climber belonging to the Vitaceae family, is well known in Thailand as "Phetsangkhat" [6]. It is used in Indian traditional medicine as a component of a poultice to treat bone fractures and swelling [7]. A number of pharmacological studies of CQ related to bone metabolism have been reported. Alcoholic CQ extracts possess bone fracture healing activities in animal models [8-13]. The ethanolic extract and the petroleum-ether fraction of CQ have been shown to possess antiosteoporotic activity in ovariectomized rats [14-16], and the petroleum-ether fraction has been shown to stimulate the development of fetal bone growth during the intra-uterine developmental stage in rats [17].

Fresh CQ stem used as a paste to cover the fracture area of patients suffering from broken bones has been shown to reduce the total time required for fracture healing and to increase the rate of clinical improvement of symptoms, such as pain, tenderness, and swelling, compared to the standard technique alone [18]. *In vitro* studies have shown that the bone protective effect of CQ extracts involves the stimulation of all processes in osteoblast development, including proliferation, differentiation, and mineralization [19, 20].

The CQ extracts have been shown to have very low toxicity. Neither acute [14, 21-23] nor sub-chronic [24] toxicity were produced by alcoholic extracts of CQ given orally to Wistar rats at doses of 5 and 3 g/kg body weight/day for three months, respectively. The $LD_{50}$ of the methanolic CQ root extract given to Swiss female albino mice via the intraperitoneal route was 1 g/kg body weight [25].

Sequential extraction is an extraction technique in which a series of solvents ranging from non-polar to highly polar are used to extract substances of all polarities. The obtained extracts are separated according to their polarity, making it easier to further investigate active compounds. Although the effects of CQ extract on bone have been reported by many researchers, only single-solvent extraction processes have been used. Thus, sequential extracts were investigated in this study. Based on the fact that this plant is used as a poultice to cover bone fractures to induce a healing effect [7], CQ juice may be the most representative of the traditional method of use. Therefore, this experiment aimed to investigate the effect of the sequential extracts and freeze-dried juice of CQ on bone metabolism using the ovariectomy-induced osteoporotic mouse model.

## Materials and methods

### *Plant material and extract preparation*

The fresh whole plant and dried stem of *Cissus quadrangularis* L. was obtained from Dong Bang village, an area in which this medicinal plant is cultivated for use at Chao Phya Abhaibhubejhr Hospital, Prachinburi province. The plant was authenticated by Prof. Dr. Wongsatit Chuakul and a voucher specimen was deposited at the Mahidol University Herbarium (PBM), Pharmaceutical Botany Department, Faculty of Pharmacy, Mahidol University.

Fresh whole plants were cleaned under running tap water, chopped into small pieces and minced using a homogenizer to obtain the fresh juice. The juice was centrifuged at 20°C, relative centrifuge force 3300g, for 20 minutes. The supernatant was separated and freeze dried.

Cleaned CQ stems were dried in a solar-drying house at 45 to 55°C for two to three days and then dried at 70-80°C for three hours. Next, the dried stem was sequentially extracted with hexane, dichloromethane (DCM), and ethanol using a Soxhlet apparatus for 24 hours (approximately one siphon action/hour). Then the aqueous extract was obtained by boiling the residue two times in distilled water (one hour after the start of boiling per time). The organic solvent extracts were evaporated in a rotary evaporator, and the aqueous extract was freeze dried. The yields were 2.25%, 1.32%, 6.74%, 5.59%, and 1.60% for hexane, DCM, ethanol, aqueous extracts, and freeze-dried juice, respectively. All of the extracts and the freeze-dried juice were kept at -20°C until solutions were prepared for analysis.

To prepare the test solutions, the extracts and freeze-dried juice of CQ were suspended in 10% olive oil in 1% carboxymethylcellulose (CMC) solution. 17-β estradiol acetate (Vetranal®), $E_2$, was prepared at a concentration of 10 μg/ml using 5% dimethyl sulfoxide (DMSO) in distilled water, and this solution was sterilized by filtering through a 0.22 μm membrane filter. The solutions were stored in an air-tight light-protected glass container at 4°C. Solutions more than one week old were discarded.

### *Animals*

Five-week-old ICR mice were purchased from the National Laboratory Animal Centre of Mahidol University, Salaya, Nakhon Pathom, Thailand. The animals were acclimatized to the experimental animal room for seven days; this room was kept at a temperature of 25□2□C with 60□10% relative humidity and a 12-hour light/dark cycle. Normal food and water were supplied *ad libitum*. The study protocol was approved by the Institutional Animal Care and Use Committee of Faculty of Pharmacy, Mahidol University (No. PYT 004/2552) and performed under the ethical principles and guidelines for the use of animals established by National Research Council of Thailand.

### *Bone formation assay*

The six-week-old mice were randomly divided into eight groups (three mice/group/cage). All mice underwent the same surgical procedure. Bilateral ovaries were removed from all mice except those in the sham-operated group to induce osteoporosis, and after surgery, the mice were returned to their cages for 14 days prior to the start of the experimental treatment. Group 1 mice underwent sham surgery and served as the normal controls. Group 2 (ovariectomized (ovx)-control) was orally administered 10% olive oil in 1% CMC solution. Group 3 (positive control) was subcutaneously injected with $E_2$ at a dose of 10 μg/kg body weight (BW)/day. Groups 4 to 8 were orally treated with the hexane, DCM, ethanol, or aqueous extracts or with the freeze-dried juice, respectively, at a dose equivalent to 5g of crude powder/kg BW/day. The treatment was conducted for two months. At the end of the experiment, all mice were sacrificed, and blood was collected. Serum was obtained by centrifugation at relative centrifuge force 400g and 25°C for 10 minutes. The serum was then immediately aliquoted and stored at -80°C for biochemical assays. The right femur and tibia were cleaned and stored at -80°C for determination of BMD of the whole bone by dual-energy x-ray absorptiometry (DEXA) (Lunar PIXImus2 densitometry; software version 2.10; Lunar corp., Madison, WI, USA). Uterus and liver were cleaned and immediately weighed. The decalcified bones (lumbar spine, L1-L5, and left femur and tibia) and the internal organs (heart, liver, kidney and stomach) were kept in 10% formaldehyde for determination of histopathological changes.

### *Serum biochemical determination*

The levels of osteocalcin and TRACP5b in the collected serum of each mouse were determined using the Mouse Osteocalcin EIA kit (Biomedical Technologies Inc.) and the MouseTRAP™ Assay (IDS Ltd.), respectively.

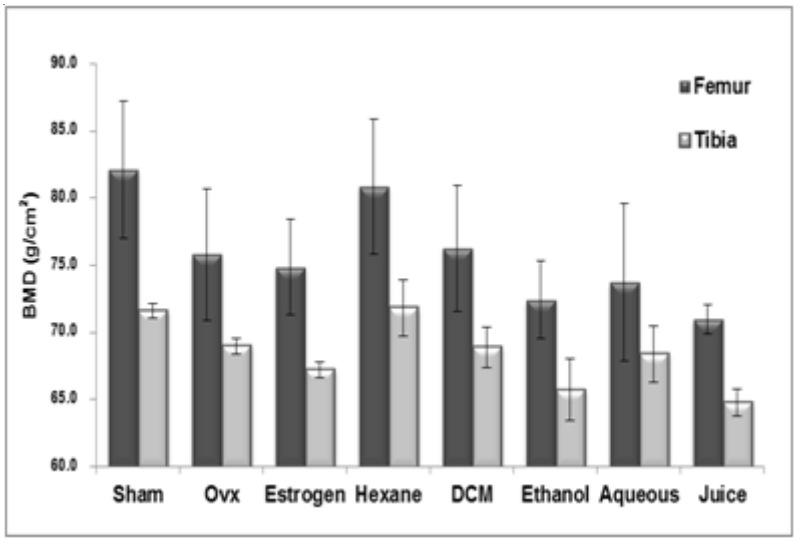


**Figure 1.** Effect of CQ extracts and freeze-dried juice on the bone mineral density (BMD) of the whole bone of femur and tibia. Administration of hexane extract increases BMDs to the same level as sham-operated group (Sham) and demonstrates the best result when compared to other CQ-treated groups. *Sham*: sham-operated group, *Ovx*: ovariectomized-control group, *Estrogen*: estradiol-treated group, *Hexane*: hexane extract-treated group, *DCM*: dichloromethane extract-treated group, *Ethanol*: ethanol extract-treated group, *Aqueous*: water extract-treated group, *Juice*: freeze-dried juice-treated group. Data are expressed as the mean±SD.

***Statistical analysis***

Numbers of animal per group were statistically calculated using equation recommended by Federer WT [26]. Data are expressed as the mean±SD. Statistical comparisons between groups were made by one-way analysis of variance (ANOVA) followed by Duncan's New Multiple Range Test (DMRT) using PASW Statistics17.0 software. A *p*-value less than 0.05 indicated a significant difference.

## Results

***Effect of CQ extracts and freeze-dried juice on bone metabolism***

After bilateral ovariectomy, the BMD of ovx-control mice was lower than the basal level of the sham-operated group, and this effect could not be overcome even by treatment with $E_2$ (**Figure 1**). The BMDs of both the femur and the tibia were elevated to the same level as that of the sham-operated group after administration of the hexane extract. This effect was not observed in the other CQ-treated groups. These results correlated with histomorphological changes of the lumbar spine. Among the CQ treatments, only the hexane extract clearly restored the trabecular bone of the lumbar spine (**Figure 2**), as observed in the $E_2$-treated group.

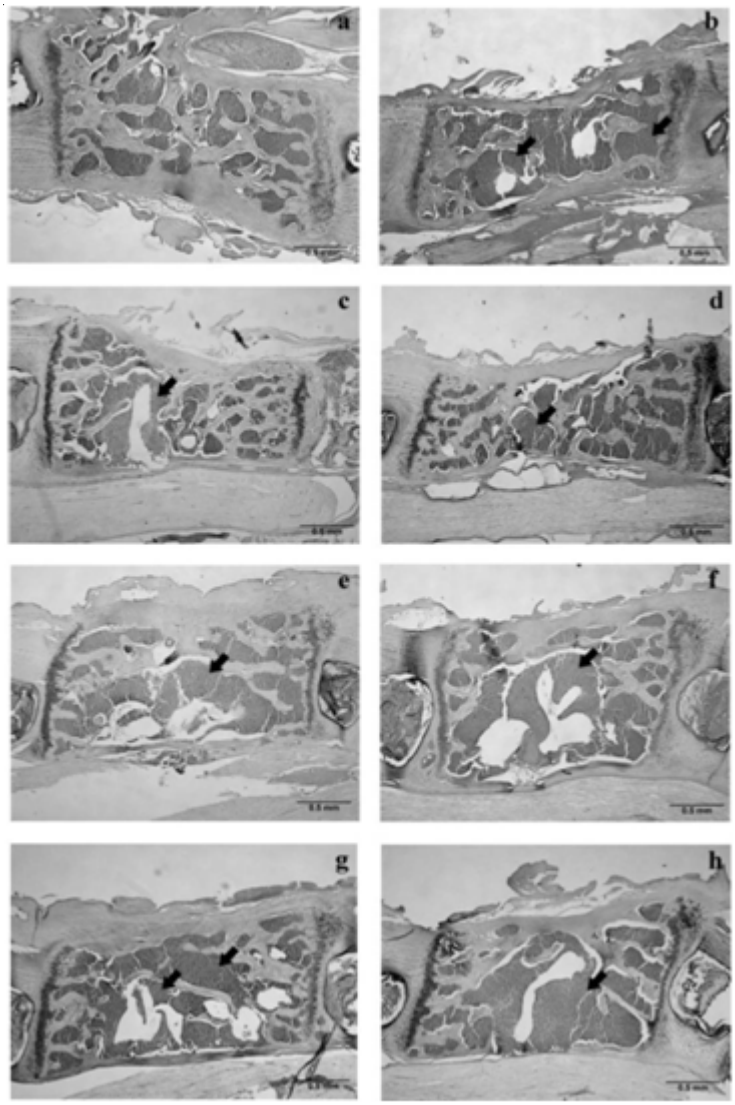


**Figure 2.** Histomorphological changes of the lumbar vertebrae (L1-L5) (sagittal plane, 40x). Decalcified lumbar vertebrae were stained with hematoxylin and eosin. The sham-operated group (**a**) shows normal architecture of the trabecular bone. The ovariectomized (ovx)-control group (**b**) shows wide area of trabecular bone loss (arrows), which is restored after administration of $E_2$ (**c**).The restoration of the trabecular bone is observed in the hexane-treated group (**d**), but not in dichloromethane (**e**), ethanol, (**f**) aqueous extract (**g**), and freeze-dried juice (**h**) groups.

The serum TRAP5b level, but not the serum osteocalcin level, of ovx-control mice was significantly reduced compared to that of the sham-operated group ($p$ <0.05). The levels of both markers were slightly increased in ovx mice when they were treated with $E_2$. The same level of TRAP5b was seen in the other CQ-extract groups, except for the hexane group, whereas the osteocalcin level was higher in the dichloromethane group, followed by the ethanol, aqueous extract and freeze-dried juice groups, respectively (**Figure 3**).

### *Effect of CQ extracts and freeze-dried juice on uterus weight change*

At the end of experiment, all mice were sacrificed, and the internal organs were removed as previously described. The cleaned uterus was immediately weighed and recorded. Bilateral ovariectomy resulted in a significant reduction in uterus weight ($p$ <0.05) as shown in **Figure 4**. The administration of $E_2$ significantly increased the uterus weight ($p$ <0.05). However, treatment with CQ extracts did not elevate the uterus weight to the same level as the treatment with $E_2$ did. Thus, the administration of CQ extracts at the given dose for 8 weeks did not affect the uterus weight of the ovx mice as much as $E_2$ did.

### *Toxicity of CQ extracts and freeze-dried juice*

None of the mice presented signs of abnormalities after treatment for eight weeks. Liver weights were not different in any of the groups (data not shown). Histopathological changes revealed casts in kidney cells and absent epithelium cells in the stomach to a mild degree. However, these changes were found in both the sham-operated and ovx-control groups. Therefore, the extract did not produce any toxicity throughout the experiment.

## Discussion

The bone turnover process was reflected by the levels of osteocalcin and TRAP5b. The increase in bone turnover rate, reflected by rising up in levels of bone marker, can be detected in both childhood and elder which are period of bone developing and declining, respectively [5]. Administration of the hexane extract resulted in the greatest reduction in both marker levels compared to the ovx-control group (**Figure 3**), representing a reduction in the bone turnover rate of this group. These results correlated with the increases in the BMDs of the femur and tibia and with the histomorphology of the lumbar spine, as described previously. Therefore, these results indicated that the hexane extract of CQ contains substances that possess beneficial effects on ovariectomy-induced bone loss in mice.

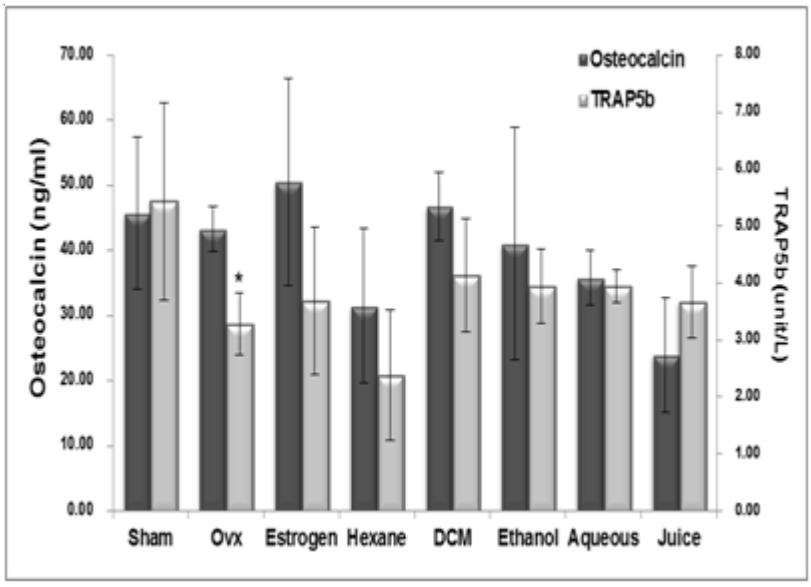


**Figure 3.** Effect of CQ extracts and freeze-dried juice on the serum levels of osteocalcin and TRAP5b: The most reduction of both enzyme levels is observed after administration with hexane extract, indicating a reduction in the bone turnover rate. *Sham*: sham-operated group; *Ovx*: ovariectomized-control group, *Estrogen*: estradiol-treated group, *Hexane*: hexane extract-treated group, *DCM*: dichloromethane extract-treated group, *Ethanol*: ethanol extract-treated group, *Aqueous*: water extract-treated group, *Juice*: freeze-dried juice-treated group. Data are expressed as the mean±SD. *$p$ <0.05 vs. Sham

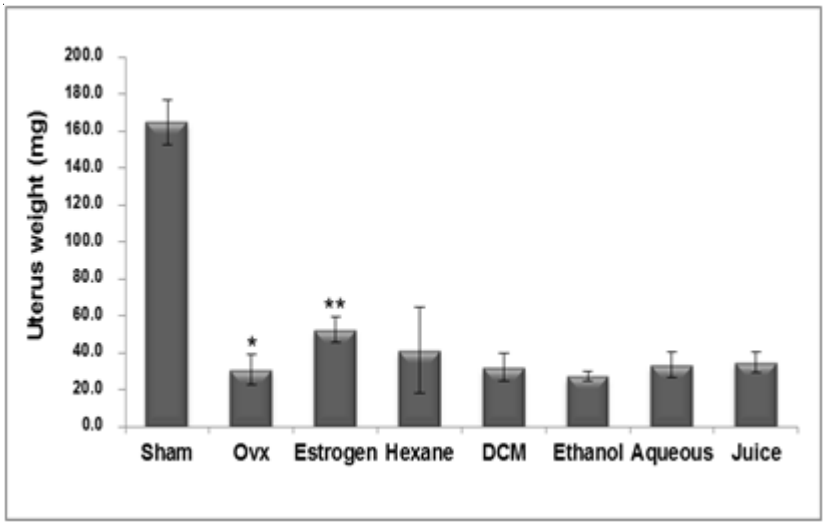


**Figure 4.** Effect of CQ extracts and freeze-dried juice on the weight (mg) of the uterus of ovariectomized mice after treatment for eight weeks: Treatment with CQ extracts and juice show no effect on reduced uterus weight when compared to ovariectomized-control group (Ovx). *Sham*: sham-operated group; *Ovx*: ovariectomized-control group, *Estrogen*: estradiol-treated group, *Hexane*: hexane extract-treated group, *DCM*: dichloromethane extract-treated group, *Ethanol*: ethanol extract-treated group, *Aqueous*: water extract-treated group, *Juice*: freeze-dried juice-treated group. Data are expressed as the mean±SD. **p* <0.05 vs. Sham, ***p* <0.05 vs Ovx.

The serum levels of TRAP5b, a bone resorption marker, were the same in the ovx-control group, the $E_2$-treated group and all CQ-treated groups except the hexane group (**Figure 3**). These results imply that these extracts, at the given dose, did not reduce the bone resorption rate. This result correlates with the results of Shirwaikar et al. [14], who found that treating ovx rats with CQ ethanolic extract resulted in the restoration of ovx-induced bone loss at a CQ dose equivalent to 5g crude drug/kg/day. However, they observed an inhibitory effect on bone resorption at higher doses. The mouse bone development can be divided into three phases similar to human; it rapidly develops during childhood until reaching a peak bone mass around five to six months of age, then steady and start to decline after 9 to 12 months of age [27, 28]. The BMDs of the femur and tibia of the sham-operated group were the highest, and this result was supported by the normal architecture of the trabecular bone of the lumbar spine (**Figure 2**). However, the highest TRAP5b and osteocalcin levels were also detected in this group, indicating that the bone turnover rate of this group is higher than those of the others (**Figure 3**). Although the BMDs and histology of lumbar spine seem like fully developed, the ICR mice in our experiment were in developing process based on TRAP5b and osteocalcin levels.

An increase in the reduced uterus weight of ovx mice after $E_2$ administration indicated that this mouse strain was affected by estrogen deficiency and that it responded an estrogen replacement therapy. Although $E_2$ administration showed restoration of the trabecular bone of the lumbar spine after ovariectomized-induced bone loss, $E_2$ administration alone cannot produce a detectable change in the BMD of femurs and tibias. This different response may be the result of the age of the mice and the dose of $E_2$ used. Mödder et al. [29] reported that three-month-old female C57BL/6 mice required a higher dose of $E_2$ than older mice to show significant increases in BMD. In this experiment, $E_2$ significantly increased the reduced uterus weight, but it resulted in only a minimal increase in both the BMD and the serum TRAP5b and osteocalcin levels compared to the results of the ovx-control group. These results may imply that administration of $E_2$ at a dose of 10 μg/kg/day had a significant effect on uterus weight. However, to produce a detectable effect on bone metabolism in this mouse strain at this age, a higher dose of $E_2$ may be required.

Although a number of pharmacological studies of CQ extracts have been reported, different extraction solvents were used. Shirwaikar et al. [14] demonstrated the antiosteoporotic activity of CQ

ethanolic extract. Ethanol is a universal solvent which could extract a wide polarity range of substances. This activity was also founded in petroleum-ether fraction obtained from partitioning an ethanolic extract with petroleum-ether which contained non-polar substances [15, 16]. These results correlated with our study that the hexane extract possessed the most protective effect on ovx-induced bone loss in mice. Thus, our results comfirmed that the pharmacologically active substances of CQ for antiosteoporotic activity should be non-polar.

## Conclusion

The results of this study indicate that the hexane CQ extract contains substances that possess a protective effect against ovariectomy-induced bone loss in mice. However, further isolation is needed to identify the pharmacologically active compounds, which will be useful for standardizing CQ raw materials and determining its mechanism of action.

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