

parthenocissine A.^[7] Other biphenyl compound-3'3',4'4'-tetrahydroxybiphenyl has been reported from the hexane extract of CQ.^[8] Recently, two new iridoids identified as 6-O-[2,3-dimethoxy]-trans-cinnamoyl catalpol and 6-O-meta-methoxy-benzoyl catalpol along with known compounds, namely picroside-I, quadrangularin A, pallidol, quercetin, quercitrin, β -sitosterol, and β -sitosterol glycoside were isolated from CQ L.^[9]

There has been a report of an acceleration of fracture repair in rats with 6 weeks injection of phytoanabolic steroid.^[10] They also reported marked increase in body weight and mucopolysaccharide content of the bone of treated animals.^[10] Subsequent to this work, few studies tried to elucidate the anabolic compounds or anabolic activity of this plant extract. An attempt to isolate anabolic steroidal substances from static tissue cultures of CQ resulted in the isolation of β -sitosterol and stigmasterol.^[11] There has been a positive regulation by ethanolic extract of CQ (CQ-EE) on proliferation, differentiation, and matrix mineralization of human osteoblast-like SaOS-2 cells.^[12] They also reported that anabolic actions of EE are mediated through increased mRNA, and protein expression of Runx2, a key transcription factor involved in the regulation of bone matrix proteins.^[12] A recent review indicated that animal and *in vitro* studies provide support for the use of *Cissus* in promoting bone fracture healing and as an antiosteoporotic. Furthermore, there are several human studies to support the use of *Cissus* extracts in weight management, but no study has been carried out in the past that demonstrate its anabolic and body building activities.^[13] However, several dietary supplements in the USA contain CQ L. extract standardized to ketosteroid content and suggest having anabolic activity.

In the present communication, we aimed at establishing the relationship between the ketosteroid content and anabolic as well as bone health-promoting activities of various *Cissus* extracts in well-established orchidectomized (ORX) rat model. Different *Cissus* extracts were prepared, and ketosteroid content was analyzed by two different commercially used methods.

MATERIALS AND METHODS

Plant materials

Stems of CQ L. (Synonyms: *Vitis quadrangularis* Wall. ex Wight; Family: Vitaceae) was obtained from the adjoining areas of Nelamangala, Bengaluru, India (13.5°N 77.23°E), in December 2012 and was identified by a certified botanist. The sample of plant material was deposited in the herbarium (NPD/820/13), The Himalaya Drug Company, Bengaluru, India.

Extracts preparation

Ethyl acetate extract was prepared by macerating powdered stems (8.8 kg, mesh size 8–10) of CQ L. with ethyl acetate (25 L \times 3) at room temperature and three different washes pooled together and concentrated *in vacuo* to yield CQ-EE (yield - 2.27% w/w). Aqueous extract (AE) was obtained from commercial batches used in Hadjod (CQ-AE, The Himalaya Drug Company) which in brief was prepared in an extractor by hot extraction with demineralized water at 80–90°C for 3 h. This process was repeated thrice, and extracted solution was pooled together, concentrated, and spray dried (yield - 10% w/w). CQ-supercritical fluid extract (CQ-SFE) was procured from Nisarga Biotech, Satara, India.

Solvents and chemicals

Solvents used were locally obtained from approved vendors and were of LR or AR grades. β -sitosterol and friedelin (Technical grade) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The testosterone - Direct ELISA kit was obtained from Diagnostics Biochem Canada Inc., (Cat. No.: CAN-TE-250).

Ketosteroid analysis

Ketosteroid analyses were performed by gravimetric methods and in the absence of any official method, following two nonofficial methods are

prevalent in commercial operation. In this study, ketosteroid analysis was performed using both methods for comparison [Scheme 1].

Method A

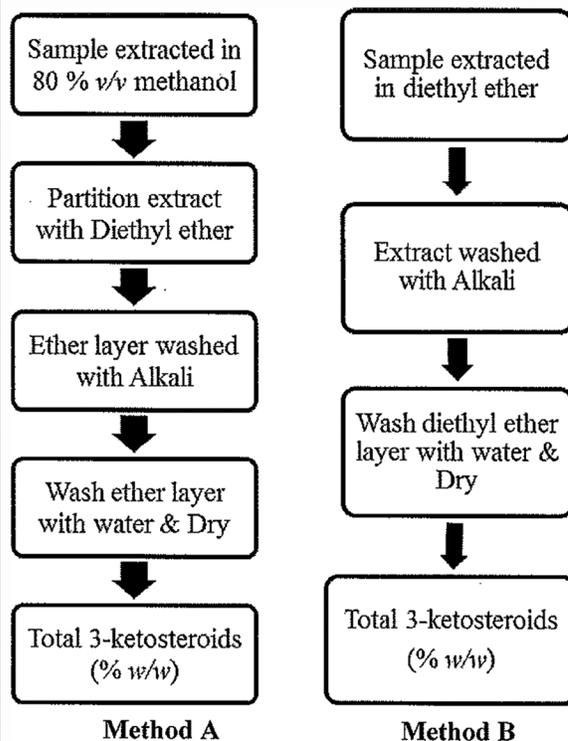
It includes dissolving 5 g of extract in 50 mL of 80% v/v methanol with warming.^[14] The residue available after filtration was rinsed with 5 mL each of 80% v/v methanol. Combined filtrates were concentrated to 25 mL and water (15 mL) was added. This was then partitioned with solvent ether which after washing was dried to constant weight which is 3-ketosteroid expressed as % w/w of dry extract.

Method B

This method is used by some of the manufacturers with slight modifications. It consists of extraction of the dry extract in diethyl ether. The filtered extract was first washed by alkali solution and then by water. The washed extract is then dried to constant weight and expressed as 3-ketosteroids on % w/w basis.

High-performance thin layer chromatography fingerprinting

All samples were dissolved in methanol (100 mg/mL) and were applied (10 μ L) on precoated aluminum silica gel plates (TLC silica gel 60 F₂₅₄ Merck, Darmstadt, Germany) using Linomat V applicator (band size; 13 mm, distance between two bands; 9 mm). The plates were then developed in a presaturated 20 cm \times 10 cm twin trough glass chamber (Camag, Muttens, Switzerland) saturated with the mobile phase using Whatman filter paper No. 1, solvent system: Hexanes:ethyl acetate (8:2 v/v). It was visualized at 254 nm and was subsequently derivatized with anisaldehyde-sulfuric acid reagent. The length of chromatogram run was 10 cm. Densitometric scanning was performed using a Camag TLC scanner III in the reflectance-absorbance mode and operated by winCATS software (V 3.15, Camag, Minneapolis, United States).



Scheme 1: Ketosteroid analysis

Anabolic effect of different solvent extracts of *Cissus quadrangularis* in orchidectomized rats

Experimental animals

Inbred 6-week-old male Wistar rats (120–140 g) were used, which were housed in standard conditions of temperature ($22 \pm 3^\circ\text{C}$), relative humidity ($55 \pm 5\%$), and light (12 h light/dark cycles) before and during the study. Normal group animals were fed with standard pellet diet (Provimi Animal Nutrition India Pvt. Ltd., India), and water *ad libitum*. The experimental protocols were approved by the Institutional Animal Ethics Committee of The Himalaya Drug Company, Bangalore, and the animals received human care as per the guidelines prescribed by the Committee for the Purpose of Control and Supervision on Experiments on Animals, The Ministry of Environment and Forests, Government of India. Animal experiments were conducted in accordance with the EEC Directive of 1986 for laboratory animal use and care. Animals were not found to show any signs of mortality pre- and post-experimentation.

Methodology

6-week-old rats (120–140 g) were ORX at the beginning of the study, and a group of sham-operated male rats were also included as intact control; the surgical procedures were carried out under aseptic conditions. The ORX animals were randomized into six groups of 10 each. Animals were maintained for 12 weeks after orchidectomy to allow for the maximum decrease in soleus muscle mass. Group I and II received vehicle and served as normal control and untreated positive (orchidectomized) control, respectively; Group III–Group V animals were treated CQ-EE, CQ-SFE, and CQ-AE, respectively, at a dose of 200 mg/kg body weight *p.o./day*, based on the animal doses used in the literature.^[13,15] Group VI animals were treated with testosterone propionate at a dose of 5 mg/kg body weight once a week subcutaneously.

Body weight and feed intake were recorded on day-1, and later once a week for 8 weeks. All the rats were maintained on normal diet, along with oral administration of respective test drugs. The treatment of respective groups was continued for 8 weeks. At the end of the treatment, blood was collected from retro-orbital sinus of overnight fasted animals and subjected for serum testosterone estimation. Animals were sacrificed, and the soleus, gastrocnemius, and levator ani muscle were dissected and weighed immediately for the estimation of anabolic activity. Femur bone was removed and subjected to bone density, strength, ash value, calcium, and phosphate. For androgenic activity, bulbus cavernous, glans penis, ventral prostate, and seminal vesicle were removed and weighed.^[16–18]

Estimation of femur bone volume, density, breaking strength, and bone mineral content

After removing the blood for biochemical analyses, the animals were sacrificed. The femur bones were removed and were freed of soft tissue using small scissors, tweezers, and cotton gauge. The bone was dried overnight in the oven and weighed. The left femur bone was subjected to the bone volume and density, which was measured by Archimedes principle as per the well-described procedure.^[19,20] Bone volume (mL) and density were then calculated using the formulae:

Bone volume = (Mass in air – Mass in water)/Density of water and bone density (g/cm^2) = Mass in air (mg)/Volume (mL).

The right femur bone of all the animals were subjected to testing bone strength and bone mineral content, briefly; the breaking strength of femur bone was estimated using hardness tester.^[15] The samples were then kept for ashing in tared fused silica crucibles, weighed, dried to a constant weight at 110°C , and ashed for 24 h at 650°C .^[21] The ash weights were determined before dissolving in 1 mL concentrated hydrochloric acid. The samples were suitably diluted with deionized water and assayed for calcium and phosphorus. Since the femur density correlates well with bone calcium content, the bone calcium was expressed in mg/cc bone volume.

Statistical analysis

All the values were expressed as mean \pm standard deviation, the results were statistically analyzed by one-way ANOVA followed by Dunnett's post test, using Prism, GraphPad 4.03 software (Prism GraphPad 4.03 Software, Inc. CA, USA) (Windows version). The value $P < 0.05$ was considered statistically significant.

RESULTS

Ketosteroids from CQ have been the focus for standardization of commercially available extract [Figure 1]. The initial reports about ketosteroids go back to 1964 when reported an oxo steroid (m.p. $134\text{--}6^\circ\text{C}$, acetyl derivative m.p. 124°C) with α , β -unsaturated ketone.^[22] Subsequently, in 1966, presences of other ketosteroids have also been reported.^[23] The structures of these ketosteroids are not known, and commercially, their analysis has been performed by gravimetric methods. The reported method by involves partition chromatography with ethyl ether, whereas other prevalent commercial method by ingredient manufacturers is direct extraction using diethyl ether^[14] [Scheme 1]. Both these methods predict the 3-ketosteroid content in the extract based on the gravimetric analysis. Due to the polarity of the solvents used in this analysis, phytosterols (along with other nonpolar/medium polar constituents) are invariably extracted in both method A and B. These methods are typically used commercially to estimate 3-ketosteroids in *Cissus* extract.

In this study, ketosteroid analysis of CQ-EE was found to be 1.40% w/w and 12.92% w/w, respectively, by method A and B. Method B yields significantly higher value of 3-ketosteroid as compared to method A. Due to the difference in the process of determination, this inherent difference [Scheme 1] in the 3-ketosteroid values is seen to be continued in the rest of the analysis [Table 1]. Due to the lack of an official method, both the results can be claimed for 3-ketosteroid content. Among the obtained three extracts, highest 3-ketosteroid content was found to be in CQ-SFE as it effectively extracts all the low polar to medium polar constituents. In high-performance thin layer chromatography fingerprint studies [Figure 2], CQ-SFE and CQ-EE show similar profile with presence of β -sitosterol in both extracts leaving only CQ-AE where no visible phytosterol pattern seen. In our studies, the standardized extracts of CQ were subjected to screening for anabolic and bone health-promoting properties in rat ORX model. In

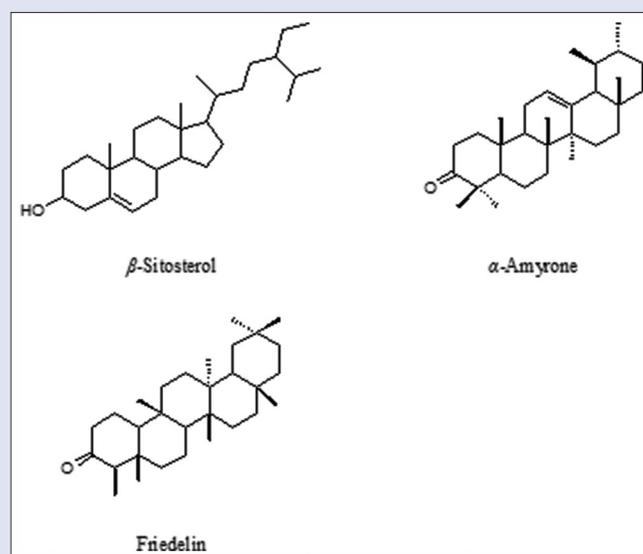


Figure 1: Important phytosterols from *Cissus quadrangularis* L.

the ORX animals, extracts/samples having anabolic activity should show corresponding increase in the weight of androgen sensitive tissues, which forms the basis of present experiment. From 3-ketosteroid analysis, the activities should be highest in CQ-SFE, followed by CQ-EE and the least

active was expected to be CQ-AE (CQ-SFE > CQ-EE > CQ-AE). However, in our studies with respect to the parameters pertaining to the anabolic and androgenic activity (absolute weight of androgen-sensitive tissues in ORX-rats) as summarized in Table 2, the results indicates that there was a trend of increase in the absolute weight of androgen-sensitive tissues which though was not found to be statistically significant. The increase in the absolute weight was appreciable in the CQ-AE treated group (CQ-AE > CQ-EE > CQ-SFE), similarly with respect to bone parameters [Table 3], a similar trend was seen. The mean bone density, strength, and calcium content were found to be highest in the group treated with CQ-AE compared to groups treated with other extracts. It was also observed that in none of the treatment group has increased the serum testosterone level to a significant extent as compared to untreated control [Figure 3]. All the above observations were in contrary to the existing understanding of ketosteroid constitution of CQ L.

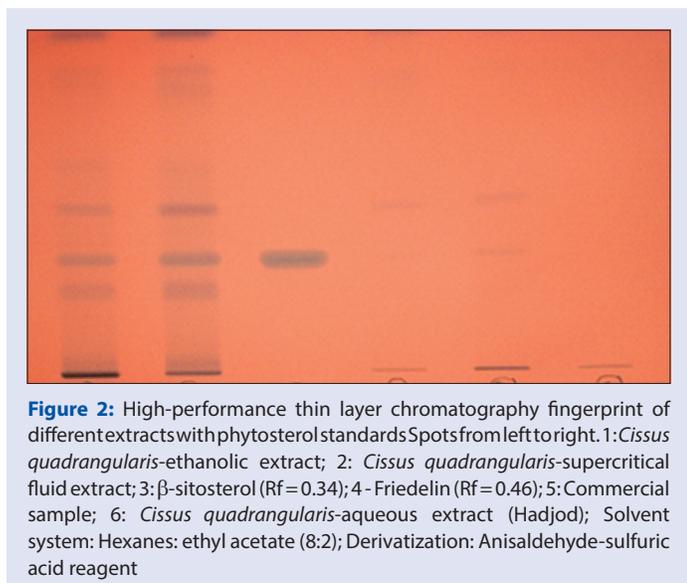


Figure 2: High-performance thin layer chromatography fingerprint of different extracts with phytosterol standards Spots from left to right: 1: *Cissus quadrangularis*-ethanolic extract; 2: *Cissus quadrangularis*-supercritical fluid extract; 3: β -sitosterol (Rf=0.34); 4- Friedelin (Rf=0.46); 5: Commercial sample; 6: *Cissus quadrangularis*-aqueous extract (Hadjod); Solvent system: Hexanes: ethyl acetate (8:2); Derivatization: Anisaldehyde-sulfuric acid reagent

DISCUSSION

Current understanding about the active principles of CQ is rather limited. Previously reported studies have shown bone healing activity to extracts ranging from hexane to aqueous. However, a recent study demonstrated that hexane extract showed beneficial effects in bone health in ovariectomized mice.^[24] Another similar study in female Wistar rats identified a phytoestrogenic fraction rich in friedelin to be active.^[25] We have also compared all the extract on TLC for phytosterol profile along with friedelin, β -sitosterol as representatives. Although above-mentioned studies were on ovariectomized animals suggestive more of estrogenic potential instead of effect on bone healing, a previous report in 2009 showed that EE increases bio-mineralization through upregulation of mitogen-activated protein kinase-dependent alkaline phosphatase activity in osteoblasts.^[26] *Cissus* is reported to contain certain stilbenes such as resveratrol, some of which are known to have a beneficial effect in bone health.^[7,27] However, the quantities reported earlier suggest that the presence of these compounds is minimal.^[7]

CONCLUSION

In light of the above estimation studies, we believe that current standardization of *Cissus* extraction “3-ketosteroids” is incorrect. We also

Table 1: Total 3-ketosteroids by method A and B (% w/w)

Sample	Method A	Method B
CQ-EE	1.40	12.92
CQ-SFE	2.90	17.0
CQ-AE	0.21	0.37

CQ-SFE: *Cissus quadrangularis*-supercritical fluid extract; CQ-AE: *Cissus quadrangularis*-aqueous extract; CQ-EE: *Cissus quadrangularis*-ethanolic extract

Table 2: Effect of CQ variants on absolute organ weight

Absolute weight (mg)	Normal control	Positive untreated control	CQ-EE (200 mg/kg)	CQ-SFE (200mg/kg)	CQ-AE (200mg/kg)	TP (5 mg/kg)
Gastrocnemius muscle	1433±91.61	1322±29.42	1375±26.66	1407±47.86	1423±28.62	1432±53.05
Soleus muscle	153.7±29.68	97.7±6.82 [†]	119.3±8.87	104.2±6.89	121.5±8.05	155.7±10.94*
LABC	374.0±13.45	42.9±3.47 ^{††}	49.6±5.98	53.3±4.91	58.5±4.61	320.9±11.83**
Seminal Vesicles	287.7±18.28	12.8±2.40 ^{††}	18.0±4.25	16.5±2.79	16.5±2.50	284.7±13.84**
Glans Penis	50.3±3.85	19.8±0.67 ^{††}	23.9±2.76	21.0±2.18	23.3±2.50	45.0±3.89**
Ventral Prostate	213.8±17.22	11.3±1.78 ^{††}	12.3±2.45	9.7±1.29	31.4±18.67	85.5±17.47**

[†]P<0.05 compared to normal control, ^{††}P<0.01 compared to normal control, *P<0.05 compared to positive untreated control, **P<0.01 compared to positive untreated control. TP: Testosterone propionate; CQ-SFE: *Cissus quadrangularis*-supercritical fluid extract; CQ-AE: *Cissus quadrangularis*-aqueous extract; CQ-EE: *Cissus quadrangularis*-ethanolic extract

Table 3: Effect of CQ variants on Bone parameters

Bone parameters	Normal control	Positive untreated control	CQ-EE (200 mg/kg)	CQ-SFE (200 mg/kg)	CQ-AE (200mg/kg)	TP (5 mg/kg)
Bone density (g/mL)	0.18±0.01	0.13±0.00 [†]	0.13±0.01	0.12±0.01	0.24±0.01**	0.25±0.01**
Bone strength (Kg/cm ²)	9.10±0.54	5.85±0.28 ^{††}	6.50±0.40	6.75±0.41	8.07±0.32*	7.77±0.54*
Bone calcium (% of ash weight)	42.80±0.58	36.05±1.03 [†]	37.41±0.87	40.56±1.11	59.19±2.03**	56.73±1.48**
Bone phosphate (% of ash weight)	10.27±1.69	5.37±0.19 ^{††}	5.35±0.12	5.77±0.19	5.95±0.29	5.74±0.31

[†]P<0.05 compared to normal control, ^{††}P<0.01 compared to normal control, *P<0.05 compared to positive untreated control, **P<0.01 compared to positive untreated control. TP: Testosterone propionate; CQ-SFE: *Cissus quadrangularis*-supercritical fluid extract; CQ-AE: *Cissus quadrangularis*-aqueous extract; CQ-EE: *Cissus quadrangularis*-ethanolic extract

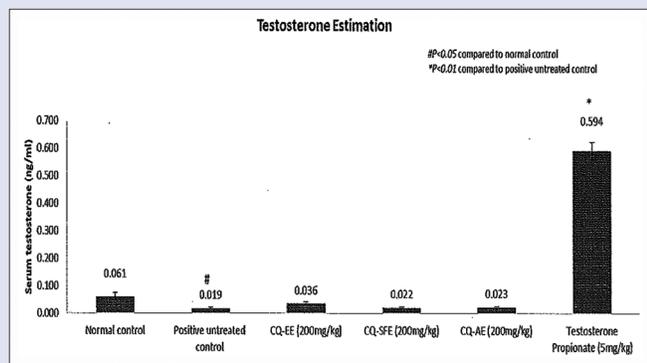


Figure 3: Effect of *Cissus quadrangularis* variants on serum testosterone levels

did not find any report suggesting the presence of androgenic steroids in this plant and hence the characterization based on “3- ketosteroids” is scientifically incorrect. This study highlights the insufficient understanding of biological active principles from CQ L. and underlines the need for extensive bioactivity guided studies.

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Conflicts of interest

The authors work for The Himalaya Drug Company, which markets CQ extract as “Hadjod.”

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