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Ethanolic extract of *Coelogyne cristata* Lindley (Orchidaceae) and its compound coelogin promote osteoprotective activity in ovariectomized estrogen deficient mice

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ABSTRACT

Coelogyne cristata Lindley (CC) family Orchidaceae is an Indian medicinal plant used for the treatment of fractured bones in folk-tradition of Kumaon region, Uttarakhand, India. In continuation of our drug discovery program, feeding of ethanolic extract to ovariectomized estrogen deficient mice led to significant restoration of trabecular micro architecture in both femoral and tibial bones, better bone quality and also devoid of any uterine estrogenicity. Subsequently, coelogin, a pure compound was isolated from ethyl acetate fraction of *C. cristata* and evaluated in in vitro osteoblast cell cultures. Treatment of coelogin to osteoblasts led to enhanced ALP activity (a marker of osteoblast differentiation), mineral nodule formation and mRNA levels of osteogenic markers like BMP-2, Type 1 Collagen and RUNX-2. Based on these results, we propose that ethanolic extract of *C. cristata* and its pure compound coelogin have potential in the management of post menopausal osteoporosis.

Introduction

Extracts and metabolites of orchids possesses useful pharmacological activities viz. antidiabetic, anticancer, anti-microbial, diuretic, anti-rheumatic, antiinflammatory, anti-cancer, anticonvulsive, hypoglycemic activities, muscle relaxant, neuroprotective and anti-viral activities (Gutierrez, 2010). Recent ethnopharmacological studies on orchidaceae revealed that a wide range of chemical compounds including alkaloids, flavonoids, glycosides, benzyl derivatives, phenanthrenes, terpenoids etc. used for the treatment of various diseases are present in a number of orchids (Hossain, 2011).

Coelogyne criststa (CC), family Orchidaceae, locally known as '*Hadjojen*' (bone jointer) is commonly used for the treatment of fractured bones in folk traditions of India (Jaiswal et al., 2004). Osteoporosis a 'silent epidemic' is alarmingly high in India. Therapeutic options of osteoporosis are limited to anti-resorptive drugs with limited efficacy in restoring bone health following bone loss. Bone forming (osteogenic/anabolic) therapy is limited to only parathyroid hormone (PTH) being extremely costly and widely not available. Therefore, finding bone anabolic agent is an unmet medical need.

As a part of our drug discovery program, we already reported C-glycosylated osteogenic flavonoids from *Ulmus wallichiana* (Rawat et al., 2009; Maurya et al., 2009). In continuation of this program, *Coelogyne cristata* extract was prepared and evaluated for its bone forming activity in vivo wherein parameters like trabecular microarchitecture, bone strength and uterine estrogenicity were studied in estrogen deficient female Balb/c mice model. Subsequently, coelogin, a pure compound isolated (Fig. 1) from ethyl acetate fraction of *C. cristata* alcoholic extract was evaluated in in vitro osteoblast cell cultures, ALP activity (a marker of osteoblast differentiation), mineral nodule formation and mRNA levels of osteogenic markers like BMP-2, Type 1 Collagen and RUNX-2.

Material and methods

Collection and processing of plant material

Fresh plant material (leave and pseudo-bulb) of CC was collected from *Bajun* forest range of Nainital district, Kumaon, Uttarakhand, India during 2012–2013 and identified by one of the author (KRA)

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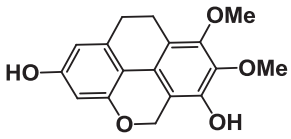

Coelogin
(7,8-dimethoxy-9,10-dihydro-5H-naphtho [8,1,2-cde]chromene-2,6-diol)

Fig. 1. Chemical structure of coelogin isolated from *Coelogyne cristata*.

according to Flora of District Garhwal (Gaur, 1999). Voucher specimen (KRA-24462) has been deposited in departmental Herbarium CSIR-Central Drug Research Institute, Lucknow. Collected plant material was washed thoroughly under running tap water, chopped and dried at room temperature. All the precautions were undertaken to avoid any types of microbial contamination. The dried material was powdered with the help of a grinder and used for further investigations.

Extraction and fractionation

Powdered material was soaked in 95% ethanol for 24 h at room temperature and percolated. This process was repeated four times. Extract was concentrated under reduced pressure using rotavapor at 40 °C and stored at 4 °C (Yield 8%).

Dried extract (80 g) was fractionated with hexane, ethyl acetate, methanol with the help of separating funnel. All the fractions were concentrated under reduced pressure using rotavapor at 40 °C. Weight of hexane, ethyl acetate, methanolic fractions was 25 g, 50 g and 5 g respectively.

Isolation of compound coelogin

Gross column chromatography was done for the isolation of compounds. Column has been packed with Silica Gel of 60-120 mesh size. Column chromatography was done with in ethyl acetate + hexane solvent system with increasing order of polarity and isolated a 9,10-dihydrophenanthrene derivative (Coelogin) from ethyl acetate fraction of *C. cristata*. Column was eluted with 10% ethyl acetate + hexane solvent system and purified by repeated column chromatography to give amorphous solid (Yield: 40 mg). TLC was run in 30% ethyl acetate + hexane solvent system to monitor purity of the compound. The compound was short UV active and showed dark red spot after methanolic H_2SO_4 spray and heating. A standardized protocol is shown here in Fig. 2.

Structural characterization of coelogin

The ESI-MS exhibited molecular ion peak $[M+H]^+$ at *m/z* 301 corresponding to molecular formula $C_{17}H_{16}O_5$. The presence of phenolic —OH and methoxy groups were indicated by IR absorptions at ν_{max} 3376.85 cm^{-1} and 1215.85 cm^{-1} respectively. It was confirmed as coelogin by its proton and carbon NMR spectral data and by comparing the spectroscopic data already reported in the literature (Majumder et al., 2001).

NMR data

^1H NMR (CD_3OD, 400 MHz) δ: 6.345 (d, 1H, J = 2.12), 6.263 (d, 1H, J = 2.12), 5.134 (s, 2H), 3.888 (s, 3H), 3.870 (s, 3H), 2.798 (s, 4H) ppm. ^{13}C NMR (CD_3OD, 100 MHz) δ 159.24, 155.29, 151.28, 145.52, 140.80, 137.77, 124.36, 117.68, 112.70, 111.99, 109.39, 102.26, 64.39, 61.41, 61.05, 28.80, 21.98 ppm; ESI-MS: 301 $[M+H]^+$

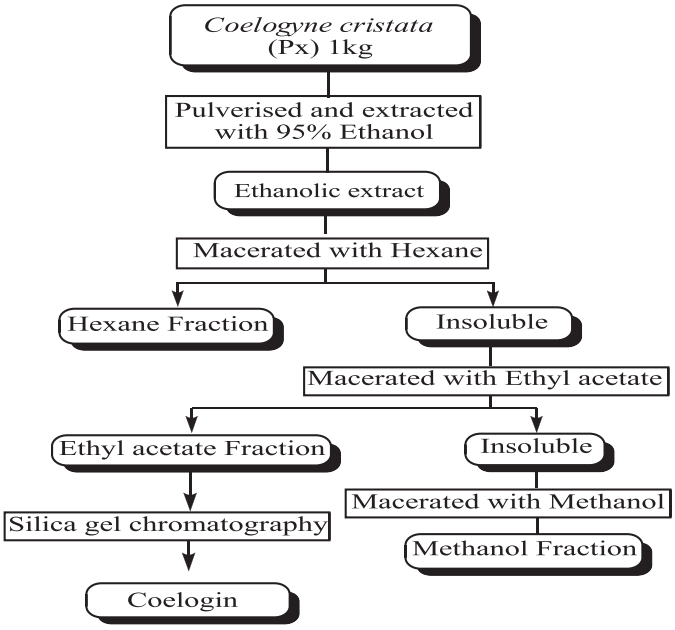

Fig. 2. Extraction and fractionation protocol used for isolation of coelogin.

Animals and experimental procedures

The study was conducted in accordance with current legislation of animal experiments (Institutional Animal Ethical Committee at Central Drug Research Institute, Lucknow). Adult female Balb/c mice weighing (typically 7–8 week) were used for the study. All mice were housed at 25 °C, in 12-h light: 12-h dark cycles.

Normal chow diet and water were provided ad libitum. Ten mice per group were taken for the study and all mice in each group were assayed and included in the statistical analyses (n = 10). For the study, animals were (Ovariectomized) Ovx and treatment with crude extract was given for four weeks. The groups were: sham-operated (ovary intact) given vehicle (gum acacia in distilled water); Ovx + vehicle; Ovx + 5.0, 10.0 and 20.0 mg/kg/1 day^{-1} CC crude extract (daily treatment by oral gavage). Initial and final body weights and uterine weight were recorded.

Microcomputed tomographic (μCT)

μCT determination of excised bones was carried out using the Sky Scan 1076 CT scanner (Aartselaar, Antwerp, Belgium) as described before (Pandey et al., 2010; Trivedi et al., 2009; Tyagi et al., 2010). Femora were dissected from the animals after autopsy, cleaned of soft tissue and fixed before storage in alcohol. The samples were scanned in batches of three at a nominal resolution (pixels) of 18 μm. Reconstruction was carried out using a modified Feldkamp algorithm using the Sky Scan Nrecon software. The X-ray source was set at 70 kV and 100 mA, with a pixel size of 18 μm. A hundred projections were acquired over an angular range of 180°. The image slices were reconstructed using the cone-beam reconstruction software version 2.6 based on the Feldkamp algorithm Skyscan). Parameters like trabecular bone volume per tissue volume (BV/TV), bone surface density (BS/BV), trabecular number (Tb.N), separation (Tb.Sp) and thickness (Tb.Th) were calculated.

Bone strength examination

Bone mechanical strength was examined by 3-point bending strength of femur mid diaphysis using bone strength tester Model TK 252C as reported earlier (Bhargavan et al., 2009). The