Phytomedicine, 2014, 21, (12), 1702–1707

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 *Coelogyne cristata* and its pure compound coelogin have potential in the management of post menopausal osteoporosis.

Key Words: Orchid, Coelogyne cristata, bone healing, Coeloginin, Osteoporosis

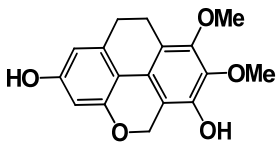
Introduction

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, *C. 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.



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*

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) 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 hours at room temperature and percolated. This process was repeated four times. Extract was concentrated under reduced pressure using rotavapor at 40 ^{0}C and stored at 4 ^{0}C (Yield 8%).
Dried extract (80g) 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 ^{0}C. Weight of hexane, ethyl acetate, methanolic fractions was 25 gm, 50 gm and 5 gm 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 standardised 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

^{1}H-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 $[M+H]^+$

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 wk) were used for the study. All mice were housed at 25 °C, in 12-hour light: 12-hour dark cycles.

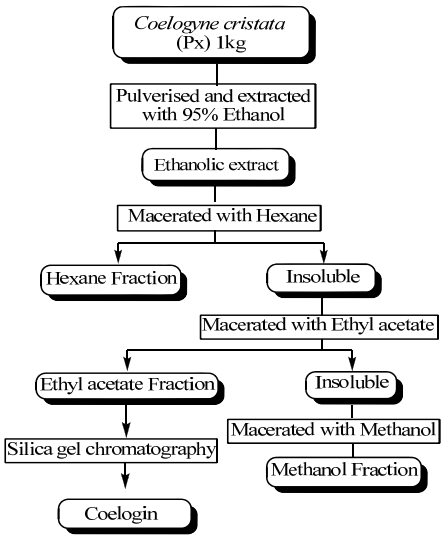


Fig. 2. *Extraction and fractionation protocol used for isolation of coelogin.*

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/1day^{-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, 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 load-displacement curves generated were used to calculate the ultimate load (N) and energy to failure (mJ).

In vitro bone formation using osteoblast culture

Mouse calvarial osteoblasts were obtained following our previously published protocol of sequential digestion (Gautam et al., 2010; Tyagi et al., 2010). Calvariae were subjected to five sequential digestions at 37°C in 0.1% dispase and 0.1% collagenase P solution. Cells released from the second to fifth digestions were collected, pooled, centrifuged, resuspended and plated in T-25cm2 flasks in α-MEM media containing 10% fetal calf serum (FCS) and 1% penicillin/streptomycin.

Alkaline phosphatase assay

For alkaline phosphatase (ALP) assay, osteoblasts were plated in 96-well (2000 cells/well) plates in growth medium with 10mM β-glycerophosphate and 50μg/ml ascorbic acid and incubated for 48 h in presence or absence of test agent. After induction, total ALP activity was determined using p-nitrophenylphosphate as substrate and quantified colorimetrically at 405 nm.

Mineralization assay

For mineralization studies, mice calvarial osteoblast cells were isolated and cultured according to a previously published protocol from our laboratory (Bhargavan et al., 2009; Gautam et al., 2010; Tyagi et al., 2010) In brief, 2.5×10^3cells/well were seeded in differentiation media with 10% FCS in presence or absence of test agent. Cells were cultured for 21 days at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, and the medium was changed every 48 h. After 18 days, attached cells were fixed in 4% formaldehyde for 20 min at room temperature and rinsed once in PBS. After fixation, the specimens were processed for staining with 40 mM alizarin red S, which stains areas rich in nascent calcium.

For quantification of alizarin red-S staining, 800 µl of 10% (v/v) acetic acid was added to each well, and plates were incubated at room temperature for 30 min with shaking. The monolayer, now loosely attached to the plate, was then scraped with a cell scraper and transferred with 10% (v/v) acetic acid to a 1.5 ml tube. After vortexing for 30 s, the slurry was overlaid with 500 µl mineral oil (Sigma–Aldrich), heated to exactly 85°C for 10 min, and transferred to ice for 5 min. The slurry was then centrifuged at 20,000×g for 15 min and 500 µl of the supernatant was removed to a new tube. Then 200 µl of 10% (v/v) ammonium hydroxide was added to neutralize the acid. OD (405 nm) of 150-µl aliquots of the supernatant were measured in 96-well format using opaque-walled, transparent-bottomed plates.

Total RNA isolation and quantitative Real-Time-PCR

Total RNA was extracted from osteoblast cells using Trizol (Invitrogen). cDNA was synthesized from 1µg total RNA with the Revert AidTM H Minus first strand cDNA synthesis kit (Fermentas, USA). SYBR green chemistry was used for quantitative determination of the mRNAs for BMP-2, RUNX-2 and Type I Col genes and a housekeeping gene, GAPDH, following an optimized protocol. Design of sense and antisense oligonucleotide primers was based on published cDNA sequences using the Universal probe library (Roche Diagnostics, USA). Primer sequences are given in table 1. For real-time PCR, the cDNA was amplified with Light Cycler 480 (Roche Diagnostics Pvt. Ltd.). The double-stranded DNA-specific dye SYBR Green I was incorporated into the PCR buffer provided in the Light Cycler 480 SYBER green I master (Roche Diagnostics Pvt. Ltd.) to allow for quantitative detection of the PCR product in a 20µl reaction volume. The temperature profile of the reaction was 95^0C for 5min, 40 cycles of denaturation at 94^0C for 2 min, and annealing and extension at 62^0C for 30sec, extension at 72^0C for 30sec. GAPDH was used to normalize differences in RNA isolation, RNA degradation, and the efficiencies of the reverse transcription.

Table 1: Sequences of Real time PCR primers

Gene name	Primer sequence	Accession number
GAPDH	F-AGCTTGTCATCAACGGGAAG R-TTTGATGTTAGTGGGGTCTCG	NM_008084.2
Bone Morph-ogenetic protein (BMP-2)	F-CGGACTGCGGTCTCCTAA R-GGGGAAGCAGCAACACTAGA	NM_007553.2

Runt-related transcription factor 2 (RUNX-2)	F-CATGTTCAGCTTTGTGGACCT R-GCAGCTGACTTCAGGGATGT	AF053956.1
Type-1 Collagen	F-CATGTTCAGCTTTGTGGACCT R-GCAGCTGACTTCAGGGATGT	NM_007742.3

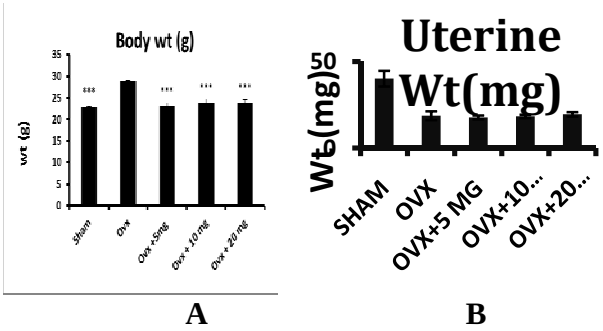
Statistics

Data are expressed as mean ± SEM. The data obtained in experiments with multiple treatments were subjected to one way analysis of variance followed by the Newman-Keuls test of significance using Prism version 3.0 software. One-way analyses of variance were performed between the sham + vehicle versus Ovx + vehicle groups and the Ovx + vehicle versus Ovx + treatment groups.

Results and discussion

Effect on body and uterine weight

CC ethanolic extract was generally well tolerated for the duration (4 week) of administration. The body weight of the Ovx group was higher than the sham group ($p < 0.001$). CC treatment resulted in significant reduction in Ovx induced gain in weight at all the three doses of 5.0, 10.0 and 20.0 mg/kg/day^{-1} ($p < 0.001$) (Fig. 3A). Efficacy of Ovx was confirmed by studying uterine parameters. Ovx resulted in reduced uterine weight compared to sham group. This ratio was not different between the Ovx + veh and Ovx mice treated with CC at 5.0 and 10.0 and 20.0 mg/kg body weight doses (Fig. 3B).

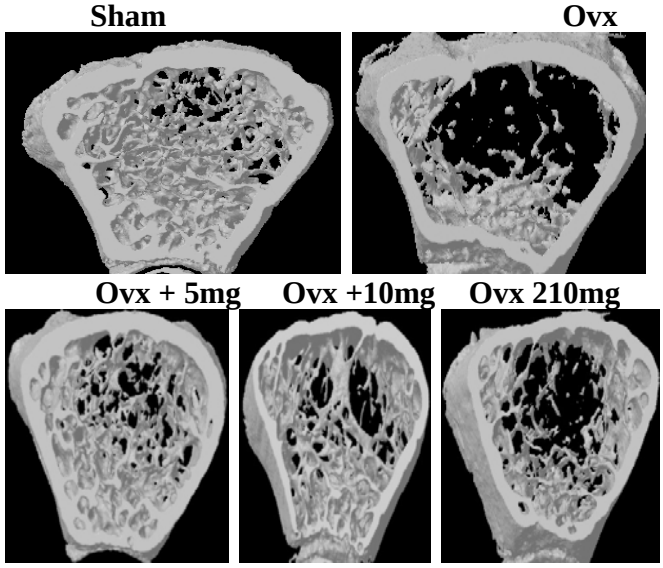


A **B**

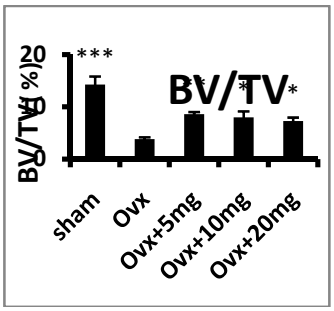
Fig. 3. Effect of CC extract on body weight (A) and uterine weight (B) after 4weeks treatment. Values represent mean ± SEM; n=10 rats/group. ***$p < 0.001$ compared to Ovx + veh.

Effect on trabecular microarchitecture in Ovx mice post treatment

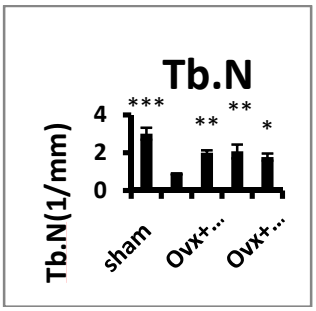
The skeletal effects of CC extract were evaluated in adult Ovx mice post one month treatment. μCT analysis of the excised femur epiphysis at the end of treatments confirmed the expected trabecular bone loss caused by Ovx compared to sham (Fig. 4A).


Sham
Ovx
Ovx + 5mg
Ovx +10mg
Ovx 210mg

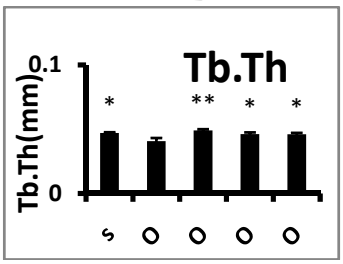
A


BV/TV
BV/TV (%)
20
0
***
**
*
sham
Ovx
Ovx+5mg
Ovx+10mg
Ovx+20mg

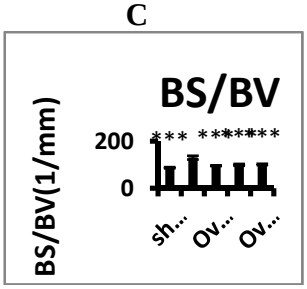
B


Tb.N
Tb.N(1/mm)
4
2
0
***
**
*
sham
Ovx+...
Ovx+...

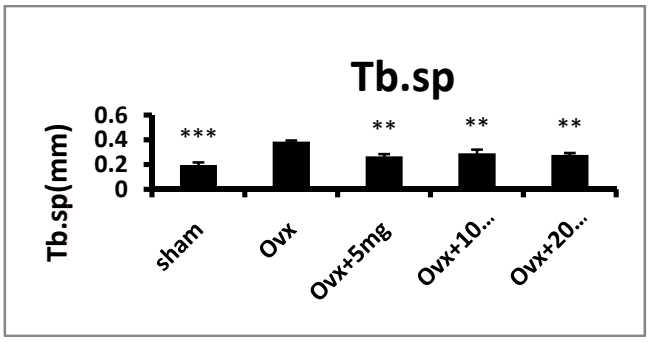
C


Tb.Th
Tb.Th(mm)
0.1
0
*
**
*
*

D


BS/BV
BS/BV(1/mm)
200
0
***
sh...
Ov...
Ov...

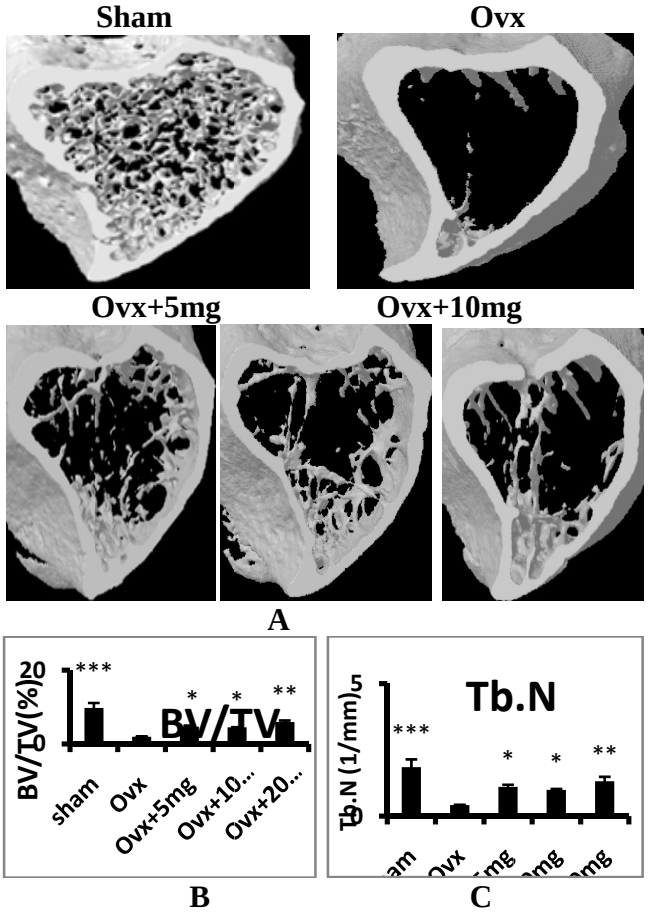
E


Tb.sp
Tb.sp(mm)
0.6
0.4
0.2
0
***
**
**
**
sham
Ovx
Ovx+5mg
Ovx+10...
Ovx+20...

F

Fig. 4 Effect of CC extract on Ovx-induced deterioration of trabecular microarchitecture in femur. After the end of treatment femurs were collected in 70% isopropanol. (A) Representative images of femur epiphysis. (B) BV/TV (bone volume/trabecular volume), (C) Tb.N (trabecular number) and (D) Tb. Th (trabecular thickness) were decreased in the Ovx in comparison to the sham and CC treatment significantly increased these parameters over the Ovx group. (E) Compared with the sham, BS/BV (bone surface density) and (F) Tb. Sp (trabecular separation) was increased in the Ovx group and CC treatment reversed this effect. N = 10 mice/group; data expressed as mean ±SD with 95% confidence interval. Statistical analysis was performed by one way-ANOVA nonparametric method followed by the Newman–Keuls test of significance using Prism version 3.0 software. $^{***}P< 0.001$, $^{**}P< 0.01$ and $^{*}P< 0.05$ compared with Ovx control.

Upon quantification of the trabecular response, the Ovx group exhibited reduced BV/TV (trabecular bone volume fraction), Tb. Th and Tb.N and increased Tb.Sp and BS/BV when compared with the sham group (Fig. 5B-F).



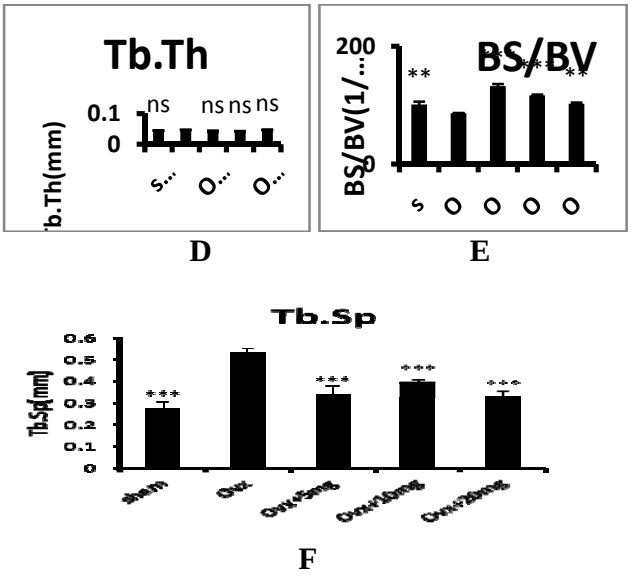


Fig. 5 Effect of CC extract on Ovx-induced deterioration of trabecular microarchitecture in tibia. After the end of treatment tibiae were collected in 70% isopropanol. (A) Representative images (B) BV/TV (bone volume/trabecular volume), (C) Tb.N (trabecular number) and (D) Tb. Th (trabecular thickness) were decreased in the Ovx in comparison to the sham and CC treatment significantly increased BV/TV and Tb.N parameters over the Ovx group. (E) Compared with the sham, BS/BV (bone surface density) and (F) Tb. Sp (trabecular separation was increased in the Ovx group and CC treatment reversed this effect. N = 10 mice/group; data expressed as mean ±SD with 95% confidence interval. Statistical analysis was performed by one way-ANOVA nonparametric method followed by the Newman–Keuls test of significance using Prism version 3.0 software. $^{***}P< 0.001$, $^{**}P< 0.01$ and $^{*}P< 0.05$ compared with Ovx control.

CC extract treatment to Ovx mice led to significant increase in BV/TV, Tb. N and Tb. Th at all three doses of 5.0 mg/kg, 10.0 and 20.0 mg/kg (Fig. 4B, C and D). In contrast, CC treatment prevented Ovx induced gain in BS/BV and Tb.Sp at all the three doses and effect at 10.0 mg/kg body weight dose equal to the sham group (Fig. 4D and E). Tibial data showed substantial deterioration in all the trabecular parameters in the Ovx group compared to the sham (Fig. 5A). The Ovx group had reduced BV/TV, Tb.Th and Tb.N and, increased BS/BV and Tb.Sp, when compared to the sham (Fig. 5B-F). CC treatment to Ovx mice led to increased BV/TV and Tb. N at all three doses of 5.0 mg/kg, 10.0 mg/kg and 20.0 mg/kg (Fig. 5B and C). Tb. Th remain unaffected in all the treatment groups (Fig. 5D). In contrast, BS/BV and Tb.Pf exhibited a decrease in the CC group in comparison to the Ovx at all the three doses and effect was equal to the sham group in 20 mg/kg dose group (Fig. 5E and F).

Effect on bone biomechanical properties

Bone strength is the measure of the quality of bone (Fig. 6) shows estimation of bone biomechanical strength in mice femoral bone. It was seen that stiffness, power and energy parameters were significantly higher in mice treated with CC extract at all the three doses of 5.0, 10.0 and 20.0 mg/kg body weight doses compared to the Ovx + vehicle group (Fig. 6. A, B and C).

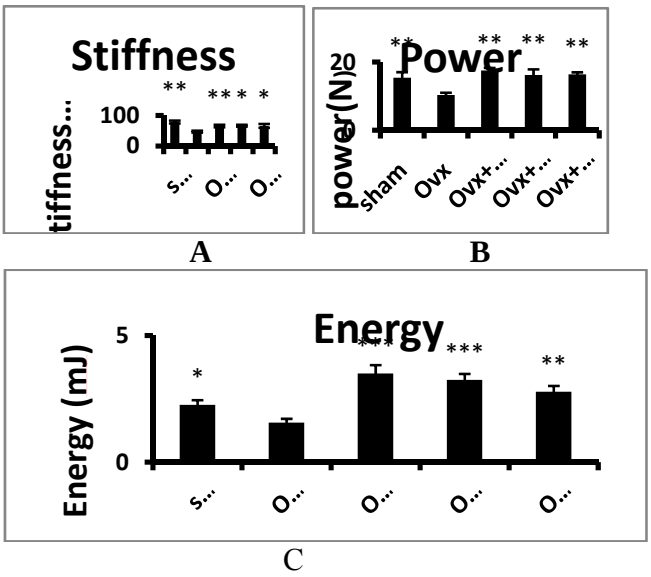


Fig. 6 A,B,C. Effect of CC extract on Ovx-induced decrease in bone strength at femur mid-diaphysis. In a 3-point bending experiment in femurs, power, energy to break and stiffness were reduced in the Ovx and CC treatment reversed this effect. N = 10 mice/group; data are presented as mean ±SEM; $^{***}P< 0.001$ compared with Ovx + vehicle group; $^{**}P< 0.01$ compared with Ovx + vehicle group $^{*}P< 0.05$ compared with Ovx control.

Effect of coelogin on osteoblast differentiation, mineralization and transcript levels of osteogenic gene markers

Subsequently, coelogin, was isolated from ethyl acetate fraction of CC ethanolic extract. In order to investigate if coelogin plays an important role in the *in vivo* bone anabolic activity of CC, osteogenic potential of coelogin was determined in calvarial osteoblast cells. The main phases in osteoblast development are differentiation and mineralization. Production of alkaline phosphatase (ALP) serves as a differentiation marker of osteoblasts. We used osteoblast ALP assay to screen the activity of coelogin following our previously published protocol (Bhargawan et al., 2009). Briefly, calvarial osteoblasts were cultured to confluence and treated with coelogin in the presence of ascorbate and glycerophosphate at concentrations ranging from 1pM to 1μM. It was observed that coelogin led to significant enhancement of ALP activity at concentrations of 10nM and 1pM. Best activity was observed at 1pM concentration (Fig. 7A).

For osteoblast mineralization, calvarial osteoblasts and bone marrow cells were cultured for 14 days in differentiation media containing 10mM β-glycerophosphate and 50 μg/ml ascorbic acid in presence or absence of coelogin. Cells were then stained with alizarin red-S and dye was extracted to quantify the extent of osteoblast mineralization. Best activity was observed at 1pM concentration (Fig 7B). In order to determine the effect of coelogin on osteogenic markers, osteoblast cells were treated with coelogin at 1pM concentration for 24 and 48h, total RNA isolated and cDNA was synthesized. This was used as a template in real time quantitative PCR.

Results show that coelogin increased the transcript levels of BMP-2, Type I Col and RUNX-2, important markers of osteoblast differentiation, at time point of 48h (Fig. 7C, D and E).

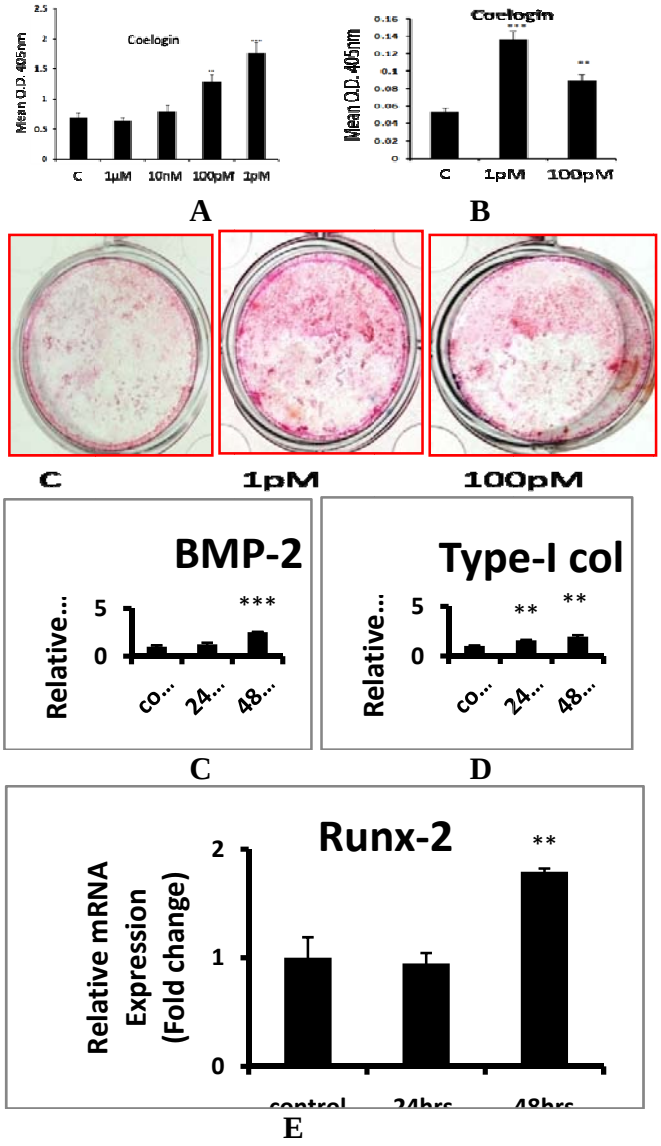


Fig. 7. Effect of coelogin on osteoblast differentiation. (A) Mice calvarial osteoblasts (2 X 10^3 cells) were seeded in 96-well plate and treated with different concentrations of coelogin for 48h. ALP activity was quantified spectrophotometrically at 405nm. (B) Effect of coelogin on mineralization of mice calvarial osteoblasts. 2x10^3 osteoblast cells were seeded in 12-well plates and exposed to different concentrations of coelogin for 14 days. At the end of the incubation, cells were fixed and stained with alizarin red-S. Stain was extracted and O.D. measured colorimetrically as described in material and method. Values are obtained from three independent experiments in the replicate of six/treatment point and expressed as mean ±SEM; **P<0.01 and ***P<0.01compared with vehicle. (C) Effect of coelogin on mRNA expression of BMP-2, (D) Type1 Col and (E) Runx-2. Osteoblast cells were cultured with or without coelogin and RNA was collected at 24 and 48h time points. qPCR for BMP-2, Type 1 Col and Runx-2

was performed as described in materials and methods. Data shown as mean±SEM; n=4; $^{**}P < 0.01$, $^{***}P < 0.001$ compared with vehicle treated cells.

Discussion

Osteoprotective properties of CC ethanolic extract was evaluated in Ovx mice model where bone loss accelerates as a result of estrogen deficiency. Our results demonstrated that CC extract at doses of 5.0, 10.0 and 20.0 mg/kg body weight exhibited significant osteoprotective effects. Importantly, extract was devoid of any uterine estrogenicity at any of these doses. The preservation of trabecular micro architecture significantly adds to bone strength and may cut down fracture risk significantly (Legrand et al., 2000). Restoration of the micro architecture network is necessary to assess the true impact of treatment of an osteoprotective agent on the quality of trabecular bones because these bones are more readily lost as a result of E2 deficiency in rodents and mice (Lane et al., 2002). CC exhibited improved trabecular response in femur bones compared to Ovx mice at all the three doses. There was an increase in trabecular bone volume fraction (reflecting a denser trabecular network), trabecular thickness and trabecular number by CC extract with a corresponding reduction in trabecular spacing and bone surface density. Remarkably, the indices that serve as the surrogate of biomechanical strength, like Tb.pf were comparable to the sham level by CC extract treatment. CC extract also restored the micro architectural deterioration resulting from ovariectomy in tibial bones where it led to increased trabecular bone volume and number and reduced BS/BV and Tb. Pf. Additionally, CC extract treatment led to improved biomechanical properties as exhibited by increased stiffness, power and energy at all the three doses in femoral bones compared to untreated Ovx animals. Since osteoporotic compression fracture correlates with the mechanical characteristics of trabecular bone, our data suggest that CC extract could effectively reduce the risk of this type of fracture by improving trabecular micro architecture in postmenopausal women.

Coelogin treatment to calvarial osteoblasts led to enhanced ALP activity (a marker of osteoblast differentiation) and increased calcium nodule formation compared to control untreated cells. Besides, coelogin also increased the transcript levels of osteogenic gene markers like BMP-2, Type I Col and RUNX-2. Together these studies show the osteogenic potential of CC extract in the management of postmenopausal osteoporosis. Future studies with coelogin in osteopenic mice model would further strengthen its potential as anti-osteoporosis agent.

Conclusion

In conclusion, our experimental results suggest that both ethanolic extract and Coelogin isolated from *C. cristata* possess significant improvement of trabecular response led to restoration of trabecular micro architecture in both femoral and tibial bones in ovariectomized estrogen deficient mice along with the biochemical strength. Our study also supports the use of CC for the treatment of fractures healing as claimed by traditional practitioners. The identified bioactive compound may serve as the starting point for design and development of pharmaceutical products not only to reduce fracture risk but also for the management of postmenopausal osteoporosis. Further work is in progress in our laboratory on these aspects.

Acknowledgements

Authors are thankful to the Director, CSIR-CDRI for constant encouragement for the program on natural products research. Chetan Sharma acknowledge the ICMR, New Delhi for Fellowship. Department of SAIF is also duly acknowledged for providing facelities. The CSIR-CDRI Communication No. is 8748.

Supporting information

All the spectral data of the isolated compound, Coelogin and the results for osteoprotective activities are available online at www.sciencedirect.com

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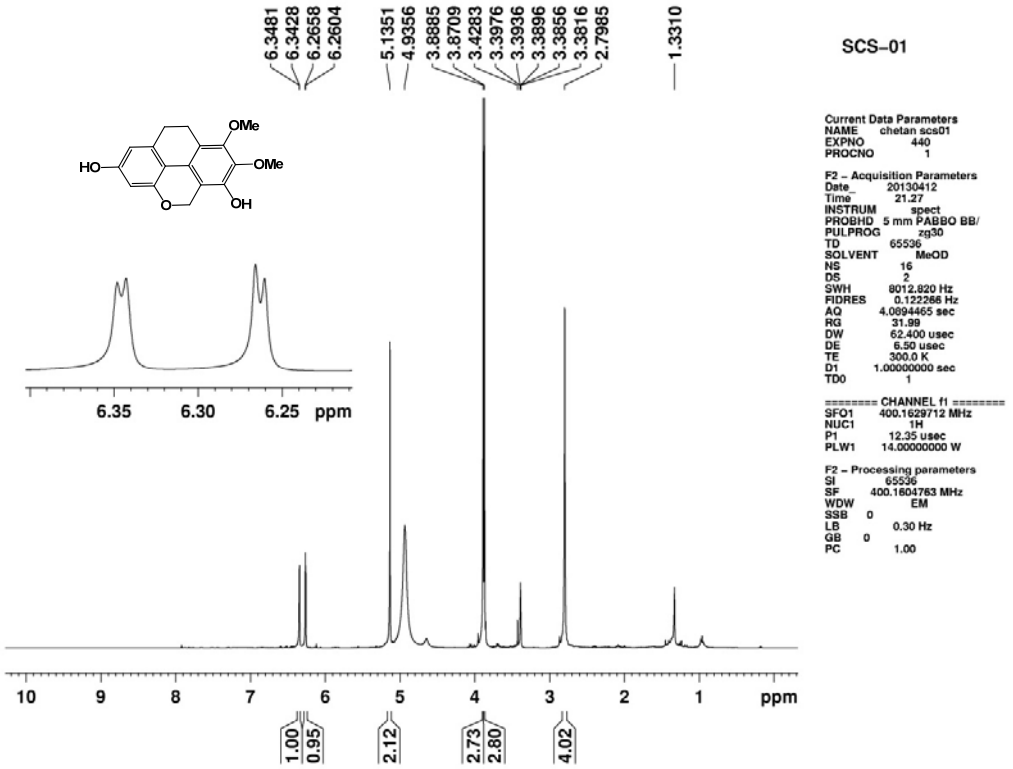
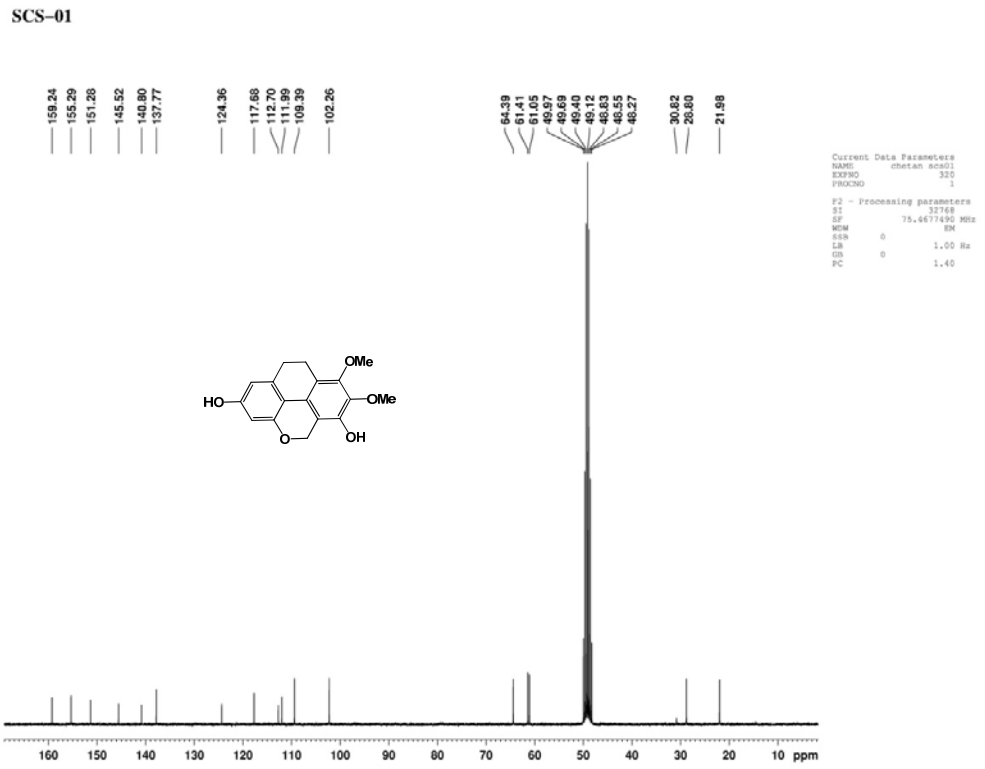
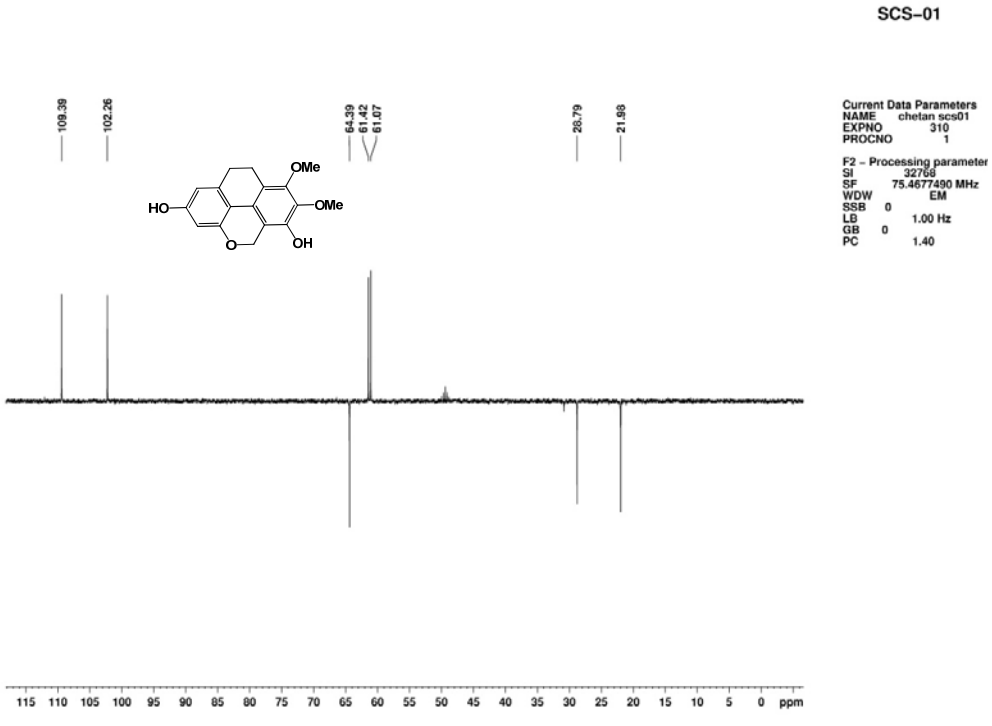
Supporting Information



^{1}H-NMR spectrum of Coelogin(CD_3OD, 400 MHz)

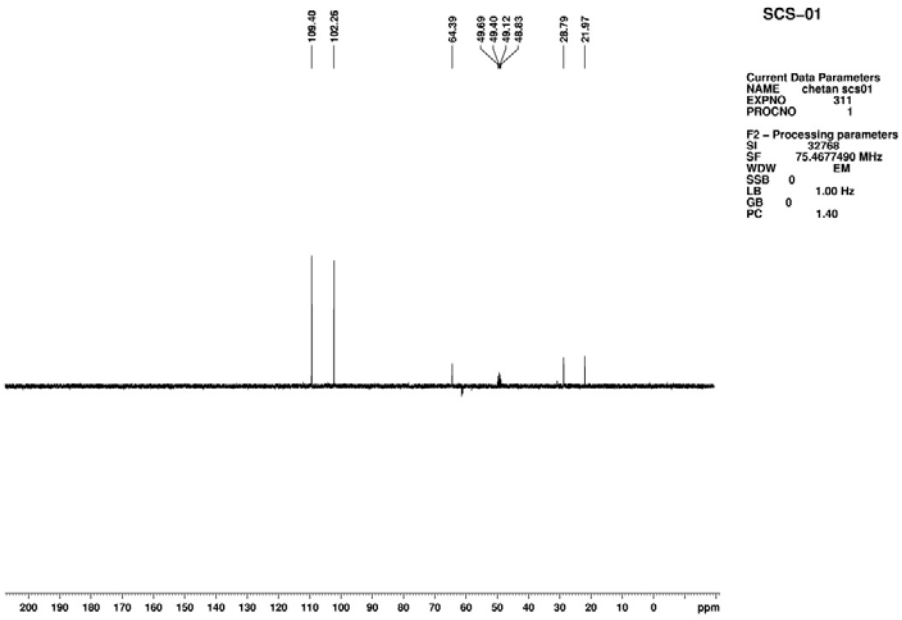


^{13}C-NMR spectrum of Coelogin (CD_3OD, 100 MHz)

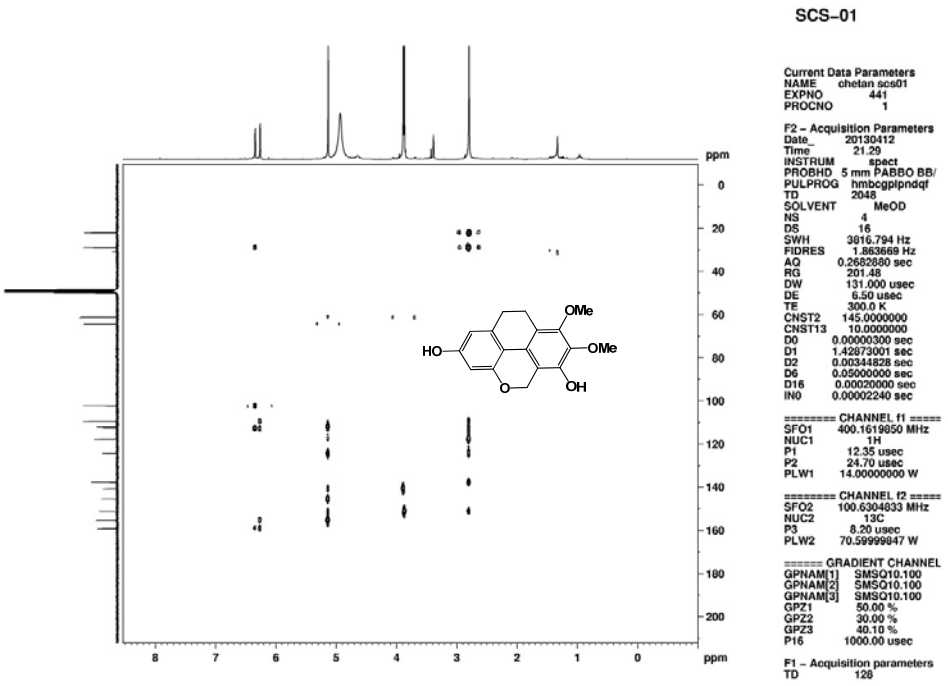


Phytomedicine, 2014, 21, (12), 1702–1707

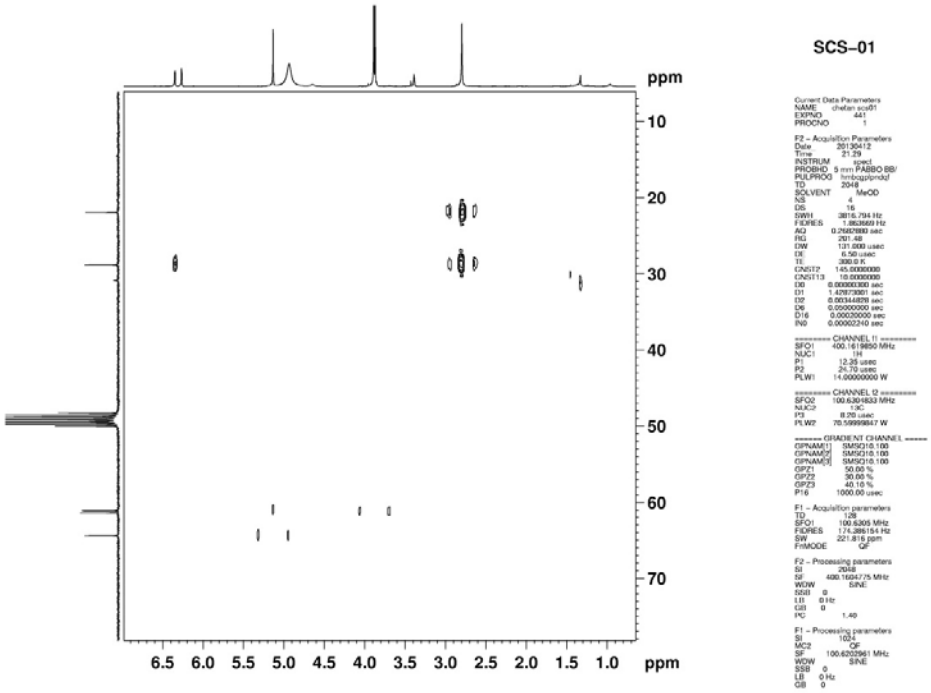
DEPT 135 spectrum of Coelogin (CD_3OD, 75 MHz)



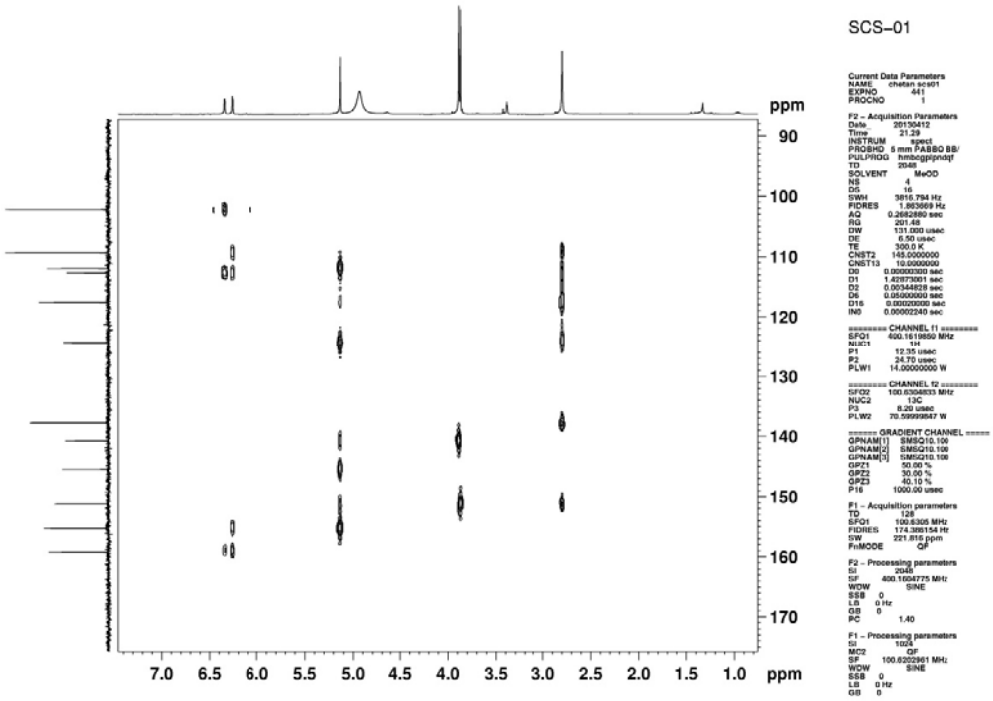
DEPT 90 spectrum of Coelogin (CD_3OD, 75 MHz)



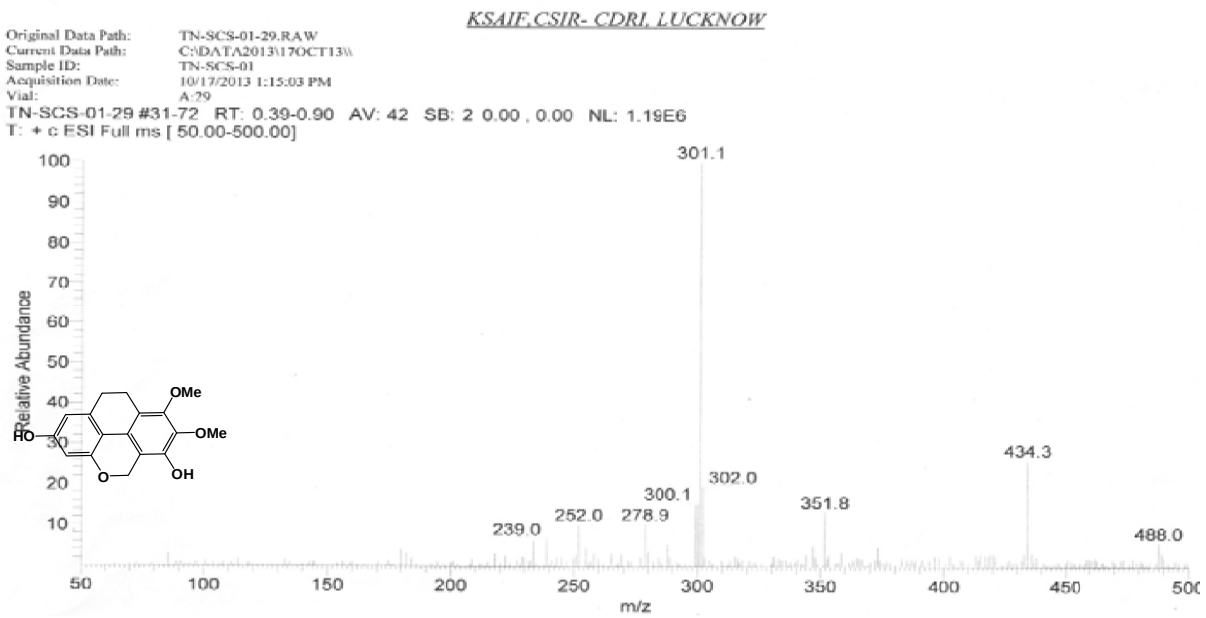
HMBC spectrum of Coelogin(CD_3OD, 100 MHz)



HMBC spectrum (Upper expansion) of Coelogin(CD_3OD, 100 MHz)



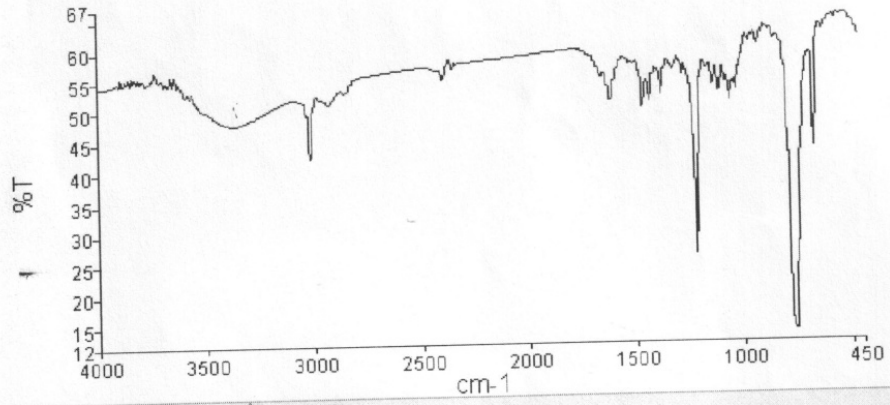
HMBC spectrum (Lower expansion) of Coelogin(CD_3OD, 100 MHz)



ESI-MS spectrum of Coelogin

PerkinElmer Spectrum Version 10.03.06
Wednesday, April 17, 2013 10:33 AM

Spectrum Graph



Name	Description
Administrator 3076	Sample 3076 By Administrator Date Wednesday, April 17 2013

Peak Table Results

SpectrumName
Administrator 3076

Sample No - SCS-1.

PeakName	X	Y
22	669.08	43.38
21	757.68	13.11
20	930.13	60.68
19	1022.46	53.33
18	1053.71	51.48
17	1106.85	52.95
16	1138.66	54.09
15	1215.85	26.11
14	1280.53	55.91
13	1319.91	56.57
12	1369.69	52.46
11	1425.92	51.34
10	1464.03	50.32
9	1608.91	51.55
8	2352.93	57
7	2400.14	55.38
6	2932.69	51.29
5	3019.43	42.65
4	3376.85	48.07
3	3660.14	54.72
2	3680.79	54.64
1	3788.1	54.93

IR Spectrum of Coelogin