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Potential osteogenic activity of ethanolic extract and oxoflavidin isolated from *Pholidota articulata* Lindley ☆

Chetan Sharma [a], Manisha Dixit [b], Rohit Singh [c], Manali Agrawal [c], Mohd Nizam Mansoori [b], Jyoti Kureel [b], Divya Singh [b], Tadigoppula Narender [c,**], Kamal Ram Arya [a,*]

[a] Botany Division, CSIR-Central Drug Research Institute, Lucknow 226031, Uttar Pradesh, India
[b] Division of Endocrinology, CSIR-Central Drug Research Institute, Lucknow 226031, Uttar Pradesh, India
[c] Medicinal & Process Chemistry Division, CSIR-Central Drug Research Institute, Lucknow 226031, Uttar Pradesh, India

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ABSTRACT

Ethnopharmacological relevance: Pholidota articulata Lindley (PA) locally known as *Hadjojen* (bone jointer) belongs to family Orchidaceae is used for healing fractures in folklore tradition of Kumaon region of Uttarakhand, Himalaya, India. Bone is a dynamic organ and is constantly being remodeled in order to facilitate growth and repair. This process requires the involvement of bone forming osteoblast and bone resorbing osteoclast cells, which function in generating and mineralizing bone, giving strength and rigidity to the skeletal system. Present study was aimed to determine the therapeutic potential of ethanolic extract of PA and its isolated compound oxoflavidin, by characterizing their fracture healing properties.

Materials and methods: Ovariectomized (Ovx) estrogen deficient adult female Balb/c mice were used for in vivo evaluation of osteogenic or bone healing potential of ethanolic extract of PA. Further, its isolated compounds were tested for their osteogenic efficacy using alkaline phosphatase assay and mineralization assay in vitro in mice calvarial osteoblasts.

Results: The ethanolic extract of PA exhibited significant restoration of trabecular micro-architecture in both femoral and tibial bones. Additionally, treatment with PA extract led to better bone quality and devoid of any uterine estrogenicity in ovariectomized estrogen deficient mice. One of the isolated compound, oxoflavidin enhanced ALP activity (a marker of osteoblast differentiation), mineral nodule formation and mRNA levels of osteogenic markers like BMP-2, Type 1 Collagen, RUNX-2 and osteocalcin.

Conclusion: These results warrant that ethanolic extract of PA and it's pure compound oxoflavidin have fracture healing properties. The extract and oxoflavidin exhibit a strong threapeutical potential for the treatment and management of postmenopausal osteoporosis.

1. Introduction

Orchids contains a wide range of bioactive compounds viz. alkaloids, flavonoids, glycosides, benzyl derivatives, phenanthrenes, terpenoids etc. used for the treatment of various diseases (Hossain, 2011). Genus *Pholidota* has nearly, 46 species widely distributed all over the world, only 5–6 species of them have been investigated in some scientific details (Gaur, 1999; Bandi and Lee, 2011). *Pholidota chinensis* Lindley and *Pholidota yunnanensis* Rolfe were shown to be rich source of stilbenoids and also possesses sedative and anticonvulsant activities (Bandi and Lee, 2011). *Pholidota articulata* Lindley (PA) is distributed throughout montane to submontane zones from Uttarakhand Himalayas (Kumaon and Garhwal) to Aruunanchal Pradesh and Indo-China to Malaysia (Gaur, 1999). Poultice made from PA locally known as *Hadjojen* (bone jointer), is one of the most common ailment used for healing fractures in folk tradition of Kumaon, Uttarakhand, India (Jalal et al., 2009; Sharma et al., 2014a, 2014b). In Ayurvedic formulation, it is referred as "*Jivanti*" and used as tonic (Ballabha et al., 2013).

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* Correspondence to: Botany Division, CSIR-Central Drug Research Institute, Sector-10, Jankipuram Extension, Sitapur Road, Lucknow-226031,Uttar Pradesh, India. Tel.: +915222772450.
** Corresponding author.
E-mail addresses: chetancdri12@gmail.com (C. Sharma), manishadixit32@gmail.com (M. Dixit), rohitsingh_chem@rediffmail.com (R. Singh), manaliagrawal26@gmail.com (M. Agrawal), nizamali786@gmail.com (M.N. Mansoori), Jyotik.biotech@yahoo.com (J. Kureel), divya_singh@cdri.res.in (D. Singh), t_narendra@cdri.res.in (T. Narender), aryakr@rediffmail.com (K.R. Arya).

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system (Parfitt, 1984). Osteoporosis is one of the diseases which results from an imbalance in remodeling and is characterized by low bone mass, micro-architectural deterioration and bone composition changes ultimately leading to fracture (Raisz, 2005). Phytoestrogens are a group of natural compounds that exert estrogenic activity and are used for the treatment of menopausal disorders and have protective effects on osteoporosis and cardiovascular system (Beck et al., 2005). Research findings report of 2003 from the Women's Health Initiative (Nassar et al., 2005; Canderelli et al., 2007) put a challenge for researchers to find out an alternative to hormone therapy especially for menopausal women. Thus, there has been an ongoing search in assessing the role of plant derived compounds and indigenous therapies in prevention of menopausal osteoporosis (Coxam, 2005; Sharan et al., 2010) and to identify better candidates which could deliver the ideal combination of outcomes. As a part of this program, we have isolated novel compounds ulmoside A and B from *Ulmus wallichiana* Planchon (Sharan et al., 2010; Rawat et al., 2009; Swarnkar et al., 2011) and a bioactive compounds coelogin from *Coelogyne cristata* (Sharma et al., 2014a, 2014b) with therapeutic efficacy for bone healing.

This paper describe the bone forming properties of ethanol extract of PA wherein parameters like trabecular microarchitecture, bone strength and uterine estrogenicity were studied in estrogen deficient female Balb/c mice model. Subsequently, three phenanthrene derivatives viz flaccidin (**1**), flavidin (**2**), and oxoflavidin (**3**) (Fig. 1), isolated from ethyl acetate fraction of PA were evaluated in osteoblast cell cultures for increased 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. Among the three isolated compounds, oxoflavidin (**3**), showed most promising osteogenic activity.

2. Materials and methods

2.1. Plant material

The plant material was collected during April 2010 from its natural habitat i.e. Nainital district of Kumaon region, Uttarakhand, India and identified by one of the senior author (KRA). Voucher specimen KRA- 24460 has been prepared and submitted to the departmental herbarium CSIR- Central Drug Research Institute, Lucknow, India.

2.2. Extraction

Plant material (leaves and pseudo-bulbs) was dried under shade and ground with the help of grinder. 1 kg powdered material was soaked in 95% ethanol and allowed to stand at room temperature for 24 h and percolated. This process was repeated four times. Extract was concentrated under reduced pressure using rotavapor at 40 °C and weighed. Dried extract was 80 g (yield 8%).

2.3. Fractionation of ethanol extract

80 g dried extract was fractionated by liquid–liquid partitioning. The extract was fractionated in to 3 fractions i.e. hexane, ethyl acetate and methanol by a standardized protocol. All the fractions were concentrated under reduced pressure using rotavapor at 40 °C and weighed. Weights of hexane, ethyl acetate, methanol fractions were 25 g, 50 g and 5 g respectively.

2.4. Isolation of compounds

Bulk column chromatography was done for the isolation of pure compounds. Column was packed with silica gel of 60–120 nm mesh size. Column chromatography was done with a gradient of ethyl acetate–hexane solvent system. Three phenanthrenes (flaccidin, flavidin and oxoflavidin) were isolated from ethyl acetate fraction of PA.

2.4.1. Isolation of flaccidin

It was eluted with ethyl acetate–hexane solvent system (30:70) and purified. TLC was run in ethyl acetate–hexane (40:60). It was short UV active and showed dark blue spot on methanolic H_2SO_4 spray.

2.4.2. Structural characterization of flaccidin

The ESI-MS exhibited molecular ion peak $[M+H]^+$ at *m/z* 270 corresponding to molecular formula $C_{16}H_{14}O_4$. The presence of phenolic –OH and methoxy groups were indicated by IR absorptions at ν_{max} 3401.22 cm-^{1} and 1215.66 cm^{-1} respectively. It was confirmed as flaccidin by its proton and carbon NMR spectral data and by comparing the spectroscopic data already reported in the literature (Majumder and Maiti, 1988).

2.4.3. NMR data

^{1}H NMR (CD_3OD, 400 MHz) δ: 6.532 (s, 1H), 6.185 (d, 1H, J=1.83), 6.112 (d, 1H, J=2.01), 5.009 (s, 2H), 3.668 (s, 3H), 2.686–2.581 (*m*, 4H); ^{13}C NMR (CD_3OD, 100 Hz) δ: 158.41, 154.23, 149.61, 142.82, 136.64, 129.82, 122.42, 120.43, 116.00, 112.94, 109.33, 102.13, 64.54, 61.39, 28.98 and 28.31 ppm; ESI-MS: 270 $[M+H]^+$ (yield: 0.0875%).

2.5. Isolation of flavidin

It was eluted in later fractions of ethyl acetate–hexane solvent system (30:70) and purified. TLC was run in ethyl acetate–hexane solvent system (40:60) and gives blue fluorescence under short UV and shows yellowish spot on methanolic H_2SO_4 spray.

2.5.1. Structural characterization of flavidin

The ESI-MS exhibited molecular ion peak $[M+H]^+$ at *m/z* 240 corresponding to molecular formula $C_{15}H_{12}O_3$. The presence of phenolic –OH groups was indicated by IR absorptions at ν_{max} 3400.45 cm-^{1}. It was confirmed as flavidin by its proton and carbon NMR spectral data and by comparing the spectroscopic data already reported in the literature (Majumder et al., 1982).

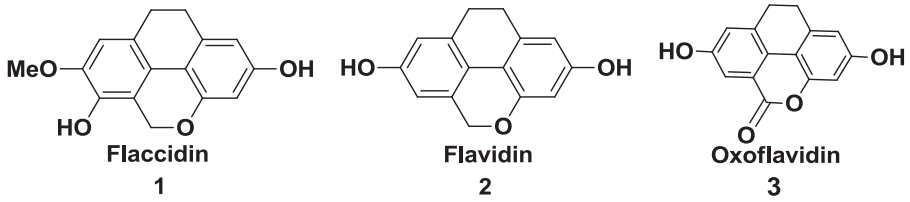

Fig. 1. Chemical structures of phenanthrenes isolated from *Pholidota articulata* Lindley (**1**) Flaccidin, (**2**) Flavidin and Oxoflavidin (**3**).