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Dysoxylum binectariferum Hook.f (Meliaceae), a rich source of rohitukine

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ABSTRACT

Rohitukine, a chromane alkaloid, is a precursor of flavopiridol, a promising anti-cancer compound. Currently in Phase III clinical trials, flavopiridol is a potent inhibitor of several cyclin-dependent kinases (CDKs). Rohitukine was first reported from *Amoora rohituka* (0.083% dry weight) followed by that in *Dysoxylum binectariferum* (0.9% dry weight), both belonging to the family Meliaceae. Here, we report incredibly high yields of rohitukine (7% dry weight) in trees of *D. binectariferum* from the Western Ghats, India. Crude extracts of the tree were found to be highly effective against ovarian and breast cancer lines tested.

1. Introduction

Rohitukine ($C_{16}H_{19}O_5N$), a chromane alkaloid, was first reported from *Amoora rohituka* (Roxb.) Wight & Arn. [1] and then from *Dysoxylum binectariferum* Hook. f. [2], both from the family Meliaceae (Fig. 1). Rohitukine exhibits both anti-inflammatory as well as immuno-modulatory properties besides acting as an anticancer compound [2]. Rohitukine is an important precursor for the semi-synthetic derivative, flavopiridol ($C_{21}H_{20}Cl\ NO_5$) [3,4].

Flavopiridol inhibits several cyclin-dependent kinases (CDKs), a family of kinases which govern progression of cells through the cell cycle, and displays unique anticancer properties [5]. In addition to directly inhibiting CDKs, flavopiridol is also known to selectively induce apoptotic cell death as well as exhibiting some anti-angiogenic properties [6]. In pre-clinical studies, flavopiridol was shown to inhibit the proliferation of a broad range of human tumor cells *in vitro* and *in vivo* and is currently under Phase II [7–11] and Phase III clinical trials, both as a single agent and in combination with other agents, particularly paclitaxel and cis-platinum [12,13]. Flavopiridol has also been shown to block human immunodeficiency virus Tat trans-activation and viral replication through inhibition of positive transcription elongation factor b (P-TEFb) [14,15]. A recent study has demonstrated the anti-fertility activity of rohitukine and efforts are being made to enhance the anti-implantation activity of rohitukine by structural modifications [16]. Though there have been efforts towards the total synthesis of flavopiridol, none has been successful, owing to the difficulty in the installation of the *cis*-alcohol functional group in the

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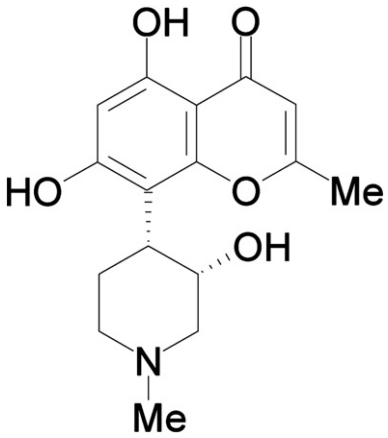

Fig.1. Structure of rohitukine.

piperidine ring [17]. Thus flavopiridol is semi-synthetically derived from rohitukine [4,18,19].

Rohitukine was first reported from *A. rohituka* (0.083% dry weight) [1] followed by that in *D. binectariferum* (0.9% dry weight) [2], both belonging to the family Meliaceae. In this study we chemically profile three populations of *D. binectariferum* in the Western Ghats, southern India for rohitukine. We have identified trees that yield about 5 to 80 fold more rohitukine than reported by Naik et al. [2] and Harmon et al .[1]. Besides, we also show the anticancer activity of the crude extracts against ovarian and breast cancer cell lines. These results have important implications for the use of the identified populations as alternative sources of rohitukine.

2. Experimental

2.1. Plant material

Stem barks of *D. binectariferum* trees were sampled from three sites, namely Jog, Kathagal and Jamboti in the central Western Ghats region of Karnataka, India. A total of 36 trees were sampled over three populations. Each tree was given a unique ID and labeled with either paint or tag. Voucher specimens (COF\DBT\WG-185-1-36) for each of the sample tree collected was deposited at the herbarium of the College of Forestry, Sirsi (University of Agricultural Sciences, Dharwad), India.

2.2. Study sites

The study was conducted in the Western Ghats, a mountain chain running parallel to the West coast of India and one of the 34 biodiversity hotspots of the world [20]. The Western Ghats contains some of the last remaining forests in peninsular India and is characterized by high levels of biological diversity. For the purpose of the study, we undertook a mapping of *D. binectariferum* in the Western Ghats between 8°N and 15°N of the equator in the states of Kerala, Tamilnadu, Karnataka and Maharashtra in South India. Study sites were short listed based on the maps developed in our previous studies.

2.3. Extraction and purification of rohitukine

2.3.1. Isolation and chemical characterization of pure rohitukine

Rohitukine was isolated from *D. binectariferum* by using the method described by Naik et al. [2]. Stem bark samples were dried to constant moisture content at 60 °C for 96 h in a hot air oven. The dried samples were ground to fine powder using a pestle and mortar. 350 g of the tissue powder was taken and extracted successively with methanol (3×500 mL, 6 h each). The extract was evaporated to remove the methanol. In the process 18.79 g of the residue was obtained and was dissolved in 200 mL water and cooled on ice. The aqueous extract was partitioned with ethyl acetate (2×100 mL) and with n-butanol (4×100 mL). All n-butanol fractions were collected and concentrated. This extract was acidified to pH 2.0 with 2 N HCl and extracted with ethyl acetate (100 mL). The aqueous layer was then basified to pH 9.0 with 29% liquid ammonia. This was extracted with n-butanol (3×100 mL) and the organic fraction was concentrated to obtain 3.192 g of a pure yellow solid which was subjected to following analysis (Supplementary data: Fig. 1S,2S,3S,4S).

LC-MS was performed on a Bruker Micro TOF-Q instrument. LC separations included reverse phase C18 column (250×4.6 mm, 5 µ), 254 nm as detection wavelength, acetonitrile and 0.1% formic acid as mobile phase and separated on a gradient mode. ^{1}H NMR and ^{13}C NMR signals were recorded in DMSO at 300 MHz for ^{1}H and 75 MHz ^{13}C. Signals were recorded in a Bruker DRX-400 Instrument. All the physicochemical parameters such as LC-MS, ^{1}H NMR, ^{13}C NMR and melting point were found to correlate well with the published parameters on rohitukine (Supplementary data: Fig. 1S,2S,3S,4S) [2,21,22]. Once the rohitukine structural identity was proved using the above parameters, for further HPLC analysis, this was used as the external standard for quantification of rohitukine in different populations of *D. binectariferum*.

2.3.2. Extraction of rohitukine from stem bark samples

Hundred milligram of fine powdered tissue was weighed into a 15 mL vial to which 5 mL of 99.9% methanol was added. After fastening the cap, the tissue was extracted in hot water bath at 60 °C for 180 min with constant shaking. After cooling the contents to room temperature, 1 mL of the extract was taken in a microfuge tube and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was filtered through a 0.22 µ filter (Tarsons, India).The filtered extract was then used for HPLC analysis. Care was exercised to ensure that the initial and final volumes of the extract were maintained constant for all samples. HPLC analysis was performed on a Shimadzu LC20AT instrument. LC separations included reverse phase C18 column (250×4.6 mm, 0.5 µ), 254 nm as detection wavelength, acetonitrile and 0.01 M KH_2PO_4 as mobile phase and separated on a gradient mode.

2.4. Cytotoxicity studies

2.4.1. Cell culture and maintenance.

Breast cancer cell lines MCF-7, T47D, MDAMB 273, and the ovarian cancer cell lines SKOV3, NCI/ADR-RES were obtained from DTP NCI. The cells were maintained in RPMI medium supplemented with 10% fetal bovine serum.

2.4.2. Analysis of chromatin condensation

Chromatin condensation analysis was done by subjecting the cells to Hoechst 33342 staining. The cells were grown on 96 well plates, after indicated treatment with extract as well as standard. Cells were stained with 0.5 μg/mL of Hoechst 33342 for 10 min and observed under UV filter sets using Nikon Epi-fluorescent microscope TE2000E. Numbers of cells with apoptotic-condensed nuclei were scored and expressed as per cent cell mortality for each treatment at least by two investigators.

3. Results and discussion

3.1. Screening D. binectariferum populations for rohitukine content

Three populations of *D. binectariferum* occurring in the Western Ghats were sampled for chemical analysis. The percent rohitukine detected in the Jog population varied from 0.15% to as high as 7.04%. In the population Jamboti, the percent rohitukine varied from 0.30% to 2.87%, while in the two individuals from Kathagal, it was 1.44% and 2.16%. The tree JB8, from Jog, was found to have the highest rohitukine content (7.04%). Repeated sampling and analysis of this tree yielded consistently higher estimates (6.77, 5.54 and 6.25%) (Supplementary data: Table 1). LC-MS analysis of representative individual JB-15 further confirmed the high rohitukine content in the sample (Supplementary data: Fig. 5S, 6S, 7S).

Overall, the mean percent rohitukine in stem bark was highest in Jog (2.43% ± 2.02%) followed by Kathagal (1.8% ± 0.5%) and least in Jamboti populations (1.63% ± 0.85%). There was no significant difference in rohitukine content across populations. To exclude the possibility that the differences among individuals might arise due to certain proximate ontogenic differences such as differences in age or girth of individuals sampled, we examined the relation between percent rohitukine accumulation and the girth size of stems. However, there seemed to be no correlation between the girth of the tree and percent rohitukine content ($r = 0.34$, $p > 0.05$).

3.2. Cytotoxicity studies

Pure rohitukine as well as crude extracts of *D. binectariferum* extract stem barks was subjected for anticancer activity against 5 human cancer cell lines. Of all the cell lines tested, *D. binectariferum* extract and pure rohitukine were found to be highly effective against NCI/Adr-Res cell line with an IC_{50} of approximately 2.8 μg/mL. Interestingly, pure rohitukine was also equally effective against another breast cancer cell line MDAMB with an IC50 of 3.0 μg/mL (Table 1).

Anticancer activity has been demonstrated for semi synthetic derivative of rohitukine, flavopiridol, through inhibition of CDKs [3,4]. Though few previous studies have directly demonstrated the anticancer activity for rohitukine, it might not be surprising considering the fact that it is now very well demonstrated that compounds that possess anti-estrogenic activity could also have inhibition of breast cancer cell proliferation by blocking entry of G_1 phase cells into the S phase, including decreased cyclin Dl expression [23]. Since rohitukine was recently established to possess anti-estrogenic effect in adult female Sprague-Dawley rats [16], it is likely that it may also inhibit the proliferation of MDAMB breast cancer cell lines through the same mechanism as it was observed for other anti-estrogens.

Table 1
IC 50 values (μg/uL) of pure rohitukine and *D. binectariferum* stem bark extract against different cancer cell lines.

Cell lines	*D binectariferum* stem bark extract	Pure rohitukine
SKOV3	15	20
T47D	>20	50
MDAMB 273	7	3
NCI/ADR-RES	2.8	2.8
MCF-7	10	15

SKOV3 — Ovarian carcinoma cells, T47D — Breast cancer cell lines, MDAMB273 — Breast cancer cell line.
NCI/ADR-RES — adriamycin-resistant cell line, MCF7 — Breast carcinoma cells.

In summary, our results have demonstrated a 6 or 7 fold higher levels of accumulation of rohitukine in *D. binectariferum* compared to that reported by Naik et al. [2]. Further still, these estimates are nearly 80 fold higher than that reported for *A. rohituka* [1]. Our results also demonstrate the anticancer activity of rohitukine on both ovarian and breast cancer lines. The identified high yielding individuals of *D. binectariferum* could serve as source material for developing nursery material for *ex-situ* plantations or *in-situ* conservation of elite material. They could also be used as a source material for developing clonal orchards as well as in developing *in-vitro* production systems. Our results have opened the distinct possibility of generating copious supplies of rohitukine, should its demand increase in the near future both due to the imminent use of flavopiridol as anticancer and anti-HIV agent as well as for other pharmacological applications [14,15].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fitote.2009.08.010.

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