Introduction

The genus *Dysoxylum*, belongs to the family Meliaceae. It is a large genus of trees and shrubs comprising more than 100 species distributed all over the world out of these about a dozen of species occurs in different parts of India chiefly in Bengal, Assam, South India, and Andaman. Endemic Indian species include *D. binectariferum*, *D. beddomei*, *D. ficiforme* and *D. malabaricum* [1]. The woods of some of them are faintly fragrant and suitable for furniture and cabinetwork. The timber obtained from these plants is of enormous economic and medicinal importance [2]. A wide range of compounds have been isolated from different species belonging to the genus *Dysoxylum*. Different species of *Dysoxylum* are used in traditional systems of medicine either as crude plant extracts or as an ingredient of several herbal preparations for the treatment of skin diseases, inflammation, cardiovascular disorder, neurological disorders, and tumor. Different compounds isolated from this genus have also showed significant therapeutic potential. Most of the plants grow predominantly in rainforests from the lowlands to mountains up to 3,000 m altitude. The indigenous people are using many plants in the genus as traditional medicine. *Dysoxylum richii*, is such an example, which is used by indigenous Fijians as a medicine to treat many diseases, such as rigid limbs, facial distortion in children, lumps under the skin, skin irritations, and as a remedy for sexually transmitted diseases. It is also reported to be used as a remedy for fish poisoning and convulsions [3].

In India, *Dysoxylum binectariferum* Hook. f grows predominantly in evergreen forest found in West Bengal, Assam, Western Ghats, Annmalai and North Kanara regions [4]. It is called by many other names in India such as Bombay white cedar, Indian white cedar, Bili devdari, Devagarige, Velley agil, Porapa and Vella agil. It is a large sized, deciduous tree with straight trunk and widespread branches. The brown color stem bark become very hard, rough and thick up to middle age.

**Taxonomic Classification**

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<tr>
<td>Binomial name</td>
<td><em>Dysoxylum binectariferum</em> Hook.f</td>
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</table>

**Traditional uses**

*Dysoxylum binectariferum* is used in various traditional systems of medicine in India, China and some other parts of the world. Different parts of the plant are used for different purposes and stem bark and leaves are used for treatment of a number of diseases. This plant has been found to possess the antibacterial, antiviral, anti-inflammatory, antioxidants, lipid lowering and cytotoxic, activities.

**Phytochemistry**

Phytochemical investigations have showed the presence of wide range of chemical compounds including alkaloids [5], tannins, triterpenes [6-8], tetranortriterpenoids [9, 10], triterpene glycosides [11] and steroids [12] in different parts of this plant. The stem bark and root bark of *D. binectariferum* contains about 15% tannins. Seeds of *D. binectariferum* contain 2.52% total ash and petroleum ether extract of the seeds give 4.54% viscous oil.

Seed coats of *Dysoxylum binectariferum* contain 40% oil. The gas liquid chromatography of oily fraction yielded essential unsaturated fatty acids such as oleic acid, linoleic acid and linolenic acid. It also yielded palmitoleic acid and eicosenoic acid as well as saturated fatty acids such as palmitic acid, stearic acid, myristic acid and arachidic acid. This oily content of seed coats of *D. binectariferum* can be used as edible oil [13].

Rohitukine, a chromone alkaloid (5,7-dihydroxy -2-methyl-8- [4-(3-hydroxy-1-methyl) piperidinyl] -4H-1-chromen-4-one, has been isolated from ethanolic extract of stem bark of *Dysoxylum binectariferum* as major active constituent and is responsible for most of the biological activities of this plant [4]. Another chromone alkaloid rohitukine N-oxide has been isolated from the butanol extract of *D. binectariferum* stem bark [14].
A variety of apo-tirucallane derivatives and teranortriterpenoids have been isolated from the different species of *Dysoxylum*, and both of these have been recognized as chemotaxonomic markers of the genus [15]. These compounds contain side chains of long hydrocarbon chains or five membered rings [16-19]. Apo-tirucallanes act as precursor for the synthesis of tetrannortriterpenoid skeleton. Loss of four carbons from the side chain generates a five membered ring, and a furan ring.

Bioactivity guided fractionation of *D. acutangulum* and *D. alliaceum* yielded a new sequiterpene (+)-8-Hydroxycalamenene which showed potent activity as fish poison [20]. 7α-acetoxyl-17 α-20S-21, 24-epoxy-apotirucall-14-ene-3 α, 23R, 24S, 25-tetraol and 7 α-acetoxyl-17 α-20S-21, 24-epoxy-apotirucall-14-en-3-one-23R, 24S, 25-triol have been isolated from the stem bark of *Dysoxylum hainanensis* [21]. A new, homoerythrina-derived alkaloid with molluscidal activity, lenticellarine was isolated from the leaves of *Dysoxylum lenticellare* and its structure was determined by spectroscopic methods [22]. Activity directed fractionation of 95% alcoholic extract of the *Dysoxylum lenticellare* leaves afforded a novel biflavonoid, robustaflavone 4',7''-dimethyl ether [23]. Aerial part of *Dysoxylum lenticellare* yielded three alkaloids 2-α-Methoxycomosivine, 2α-methoxylenticellarine and 2α-hydroxylenticellarine [24]. The nonpolar hexane fraction of stem bark of *Dysoxylum pettigrewianum* yielded 3α-hydroxy-7, 24Z-
tirucalladien-26-oic acid, Masticadienonic acid A, dysoxylic acid A and dysoxylic acid B [25].
Three new tetranortriterpenoids dysoxylumin A, dysoxylumin B, and dysoxylumin C have been isolated from aerial part of *Dysoxylum hainanense* [26].

Bioactivity directed fractionation of *Dysoxylum cumingianum* yielded six new triterpene glucosides, cumingianosides A-F. In addition two trisnor- and tetranortriterpene glucosides cumindysoside A and cumindysoside B have also been isolated from same extract [27]. Six tirucallane derived triterpenoids: 3 β, 25-dihydroxy-tirucalla-7,23-diene (1), 3β,22S-dihydroxy-tiru-calla-7,24-dien-23-one (2), 23,26-dihydroxy-tirucalla-7,24-dien-3-one (3), 22,23-epoxy-tirucalla-7-ene-3 β,24,25-triol (4), tiru-calla-7,24-diene-3 β,23-diol (5) 24,25-epoxy-3 β,23-
Dysoxylum binectariferum
dihydroxy-7-tirucallene (6), have been isolated from the stem bark of D. hainanense [28].

Dysobinin and dysobinol have been isolated from ethanolic extract of fruits of D. binectariferum [10]. Other important compounds isolated from different extracts include rechenol, rechenone, rechenoic acid, dymacrin A and dymalol. Phytochemical investigation of Dysoxylum acutangulum yielded four new chromone alkaloids chrotacumines A- D, together with known compound rohitukine.
Chrotacumines A and B have been isolated from methanolic extract of leaves where as chrotacumines C, chrotacumines D and rohitukine have been isolated from ethanolic extract of stem bark [29]. Dysoxylentin A, a 21-nortriterpenoid, containing a (Z)-2-ethylidenefuran-3(2H)-one functional group was isolated from the stem of Dysoxylum lenticellatum. It showed selective anticancer activity against human myeloid leukemia HL-60 cell lines [30].
Pharmacology

Several pharmacological studies on the different active fractions and compounds have been carried out. The reported biological activities are discussed below.

Different compounds obtained from *Dysoxylum* were evaluated for antiproliferative activity using different human cancer cell lines. Wang *et al* isolated four novel nor-dammarane triterpenoids from 70% ethanolic extract of stem bark of *D. hainanense*. These four compounds were identified as $12\beta$-O-acetyl-$15\alpha,28$-dihydroxy-$3$-oxo-17-en-20,21,22–23,24,25,26,27-octanordammanran (1), $12\beta$-O-acetyl-$15\alpha,17\beta,28$-trihydroxy-$3$-oxo-20,21,22–23,24,25,26,27-octanordammanran (2), $12\beta$-O-acetyl-$15\alpha,28$-dihydroxy-$17\beta$-methoxy-$3$-oxo-20,21,22–23,24,25,26,27-octanordammanran (3) and $12\beta,15\alpha,17\beta,28$-tetrahydroxy-$3$-oxo-20,21,22–23,24,25,26,27-octanordammanran (4). All four compounds showed significant antiproliferative activity against different cancer cell lines including SK-OV-3 (ovarian cancer), A549 (non small cell lung cancer), HCT (colon cancer) and SK-MEL-2 (skin melanoma) [31].

Tirucallane-type triterpenoids, 3$\beta$, 16$\beta$, 21$\alpha$, 25-tetrahydroxy-20, 24-cycloptirucalla-7 (8)-ene and 16$\beta$, 21$\alpha$, 25-trihydroxy-20,24-cycloptirucalla-7(8)-en-3-one isolated from *Dysoxylum lukii* showed prominent cytotoxic potential against different human cancer cell lines including MCF-7 (breast cancer), A549 (lung cancer), SGC-7901 (gastric cancer), HeLa (cervical cancer), HCT15 (colon cancer), HepG2 (hepatic cancer), SK-MEL-2 (skin cancer) and BGC-823 (gastric cancer) with IC50 value in µM range. Five other triterpenoids, isolated from the same plant- methyl 6-oxomasticadienolate; $3\beta$-hydroxytirucalla-7,24-diene-6,23-dione; (23Z)-$3\beta$,26-dihydroxy tirucalla-7,23-diene; $3\beta$,26-dihydroxytirucalla-7,24-diene-6,23-dione; $3\beta$-hydroxytirucalla-7,24-dien-23-one also showed significant cytotoxic potential against different human cancers. Five new tirucallane type triterpenoids isolated from the stem bark of *D. lukii*- cavraleone, cabraleadiol, dubione B, dysoxylumstatin A and dysoxylumstatin B were shown to exhibit moderate cytotoxic potential. Two limonoids dysoxylumstatin C and meliatoosenins B, isolated from the same extract also showed moderate cytotoxic activity. In addition, $3\beta$, 16$\beta$, 21$\alpha$, 25-tetrahydroxy-20, 24-cycloptirucalla-7 (8)-ene and 16$\beta$, 21$\alpha$, 25-trihydroxy-20, 24-cycloptirucalla-7(8)-en-3-one showed significant antimicrobial activity against different Gram-positive and Gram-negative bacteria [32].
Diterpenes dysokusone A and dysokusone B, isolated from *Dysoxylum kuskusense* showed significant cytotoxic activity against HL-60 cells with EC50 values in μM range. Dysokusone A also showed prominent cytotoxic potential against NCI-H522 and K-562 cell lines [33]. Crude ethanolic extracts of *D. binectariferum* stem bark as well as pure compound rohitukine showed potent anticancer activity against different breast cancer cell line including MCF-7 and MDAMB 273 as well as in ovarian cancer cell lines such as SKOV3, NCI/ADR-RES. It has been postulated to possess significant anti-estrogenic activity which support its inhibitory effect on proliferation of breast cancer cell through inducing cells cycle arrest [34]. Cumingianisides and triterpenes isolated from the leaves of *D. cumingianum*, showed significant cytotoxic potential [27]. Methanolic extract of the aerial part of *Dysoxylum cauliflorum* showed significant antimalarial activity, inhibiting the growth of *Plasmodium falciparum*. It also exhibited toxic effect on growth of brine shrimp [35]. Triterpenes and sesquiterpenes, isolated from the ethanolic extracts of stem bark of *D. cauliforum*, have shown prominent antiproliferative effect on NSCLC-N6 cells (a non-small cell bronchopulmonary carcinomas cell line) [36].

The ethanolic extract of stem bark of *Dysoxylum binectariferum* and its fractions have been evaluated against *Leishmania donovani*, the causative agent of visceral leishmaniasis. Ethanolic extract has been shown to exhibit potent antileishmanial activity against amastigote as well as promastigote forms in macrophage system. Its chloroform soluble fraction also showed potent activity against both promastigote and amastigote forms of the parasite. Ethanolic extract showed marginal antileishmanial activity when evaluated in *in vivo* hamster model. Chloroform fraction as well as the pure compound, rohitukine isolated from that fraction also exhibited moderate antileishmanial activity [37]. Rohitukine, obtained from bioactivity guided fractionation of the stem bark of *Dysoxylum binectariferum* showed prominent anti-inflammatory, analgesic and immunomodulatory activities [4]. The ethanolic extract of *Dysoxylum lenticellare* leaves and its active constituents showed molluscicidal activity [22]. Dysobinin, a tetrnortriterpene obtained from the alcoholic extract of air dried powdered fruits of *D. binectariferum*, has been shown to exhibit potent CNS depressant activity as well as mild anti-inflammatory potential [10]. Ethanolic extract of stem bark of *D. binectariferum* as well as its active constituent rohitukine have been shown to possess potent contraceptive activity. Oral administration of ethanolic extract prevented the postcoitum pregnancy.
The chloroform soluble fraction and its major active constituent rohitukine have shown to prevent the pregnancy at lower doses. Rohitukine has shown moderate uterotropic effect without cornification of vaginal epithelium or promoting the premature opening of vagina thought to be mediated by mobilization of tissue fluid in the uterus. Recent studies established that it is structurally similar to the known estrogen agonist 17β-estradiol, which allows it to interact with the hydrophobic pocket of estrogen receptor. However it lacks a basic side chain to interact with antiestrogen binding site of estrogen receptor thus it has no estrogen antagonistic activity.
Semi-synthetic work on rohitukine by previous workers

A semi-synthetic analog of rohitukine, flavopiridol (cis-5,7-dihydroxy-2-(2-chlorophenyl)-8-[4-(3-hydroxy-1-methyl)-piperidinyl]-1-benzopyran-4-one] has been prepared from rohitukine which showed significant antitumor activity against a wide range of human cancer cell lines including lung carcinoma, breast carcinoma as well as inhibited tumor growth in human tumor xenografts in nude mice xenograft models [38]. It has been postulated to act through inducing cell cycle arrest in G1 and G2 phases [39, 40]. It is a potent and selective inhibitor of the CDKs and is currently undergoing advanced Phase-II clinical trials for anti-cancer therapy. Recent studies have revealed that its inhibitory effect is caused due to interaction with the ATP binding site of the CDKs in the ATP-binding site, which led to the postulation that Cdk2 inhibition by flavopiridol is competitive with ATP [41].

The literature survey reveals that different *Dysoxylum* species are frequently used for therapeutic purpose all over the world. Encouraged by the use of *Dysoxylum* species in different traditional systems of medicine and research work intermittently published on it, an attempt is made towards the phytochemical and pharmacological evaluation of *Dysoxylum binectariferum* which still remains a potential source of novel molecules with diverse pharmacological activities.

**Aim and design of work**

The aim of our study is to isolate the chemical constituents as well as lead molecule, rohitukine from the stem bark of *Dysoxylum binectariferum*. Owing to the immense therapeutic potential of rohitukine, considerable interest has been focused on its semi-synthetic modification and we intended to synthesize different Sulphonyl derivatives of rohitukine in order to search for novel analogues and evaluate them for anticancer activity. Further, the lead molecule rohitukine has been evaluated for antiadipogenic and antidyslipidemic effects.
**Present study**

The stem bark of *Dysoxylum binectariferum* was collected from Sindhuburg, Maharashtra, India, and identified by the Department of Botany, CSIR- Central Drug Research Institute, Lucknow, India. A voucher specimen (CDRI plant code number 4032) has been kept in the herbarium of the Institute. The chapter deals with the isolation of compounds from 95% ethanolic extract of stem bark, semi-synthetic modification of rohitukine and biological evaluation of rohitukine as well as its analogues.

**Extraction, Fractionation, Isolation and characterization of compounds**

The dried and powdered stem bark was successively extracted with 95% ethanol by cold percolation method. Combined ethanolic extract was filtered and solvent was evaporated under vacuum below 50°C to yield a dark brown viscous mass (ethanolic extract). The crude ethanolic extract was fractionated into *n*-hexane soluble (F001), chloroform soluble (F002), *n*-butanol soluble (F003) and *n*-butanol insoluble (F004) fractions. The most active chloroform soluble fraction (F002) was taken for detailed chemical investigation. Repeated column chromatography of fraction F002 over a silica gel (100–200mesh) column afforded five compounds **DB-1** to **DB-4**. These compounds were characterized as lupeol, β-sitosterol, 7, 24-tirucalladiene, 3-oic acid and rohitukine. The compound **DB-4** was isolated in quantity and thus selected for semisynthetic modification to get new analogues (**Scheme 1**). A summary of isolation procedure is given in flow sheet 2.1 and the compounds isolated are given in table 2.1

**Table 2.1:** Compounds isolated from *Dysoxylum binectariferum* stem bark

<table>
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<th>Compound code</th>
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<th>Molecular weight</th>
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<tr>
<td>DB-1</td>
<td>C₃₀H₅₀O</td>
<td>426</td>
<td>Lupeol</td>
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<tr>
<td>DB-2</td>
<td>C₂₀H₅₀O</td>
<td>414</td>
<td>β-sitosterol</td>
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<tr>
<td>DB-3</td>
<td>C₃₁H₅₀O₃</td>
<td>454</td>
<td>7, 24-Tirucalladiene, 3-oic acid</td>
</tr>
<tr>
<td>DB-4</td>
<td>C₁₆H₁₉NO₅</td>
<td>305</td>
<td>Rohitukine</td>
</tr>
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</table>
Structure of isolated compounds

Flow sheet 2.1: Summary of extraction, fractionation and isolation procedure
All the above compounds are known and were compared with the extensive literature [4, 10, 14, 42, 43] available for their IR, mass, NMR data (\(^1\)H and \(^{13}\)C) as well as with their authentic samples on TLC.

Semi-synthetic scheme undertaken for the synthesis of novel derivatives of rohitukine

**SCHEME-1:** Synthesis of novel rohitukine derivatives

**General procedure for the synthesis of target compounds K1-K11**

Rohitukine (0.305 g, 1.0 mmol) was stirred in the pyridine- triethylamine (6:2) solution at 40\(^{0}\)C for 1 hour. Different substituted sulphonyl chlorides (1.0 mmol) were added to this stirring solution and reaction was continued for further 4 hours. After completion of the reaction (confirmation by TLC), the reaction mixtures were neutralize with 1 N HCl and extracted with DCM. Reaction mixtures were washed with water (20ml x 2), brine solution (20ml x 2) and dried anhydrous Na\(_2\)SO\(_4\). The reaction mixtures were evaporated *in vacuo* and residue were purified through column chromatography on silica gel (100-200 mesh), using hexane and chloroform in different concentrations (ranging from 10% to 80% chloroform in hexane) to give the target compounds K1 to K11 (Scheme 1). These sulphonyl derivatives were synthesized in moderate to good yield (**Table 2.2**).
Table 2.2: Percentage yield of different sulphonyl derivatives of rohitukine

<table>
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<td><img src="image" alt="R2" /></td>
<td>C_{22}H_{22}N_{2}O_{9}S</td>
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<td><img src="image" alt="R3" /></td>
<td>C_{22}H_{22}N_{2}O_{9}S</td>
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<td>76%</td>
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<tr>
<td>K-4</td>
<td><img src="image" alt="R4" /></td>
<td>C_{22}H_{22}ClNO_{7}S</td>
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<tr>
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<td>C_{22}H_{21}ClNO_{7}S</td>
<td>513</td>
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<tr>
<td>K-6</td>
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<td>C_{23}H_{25}NO_{7}S</td>
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<tr>
<td>K-7</td>
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Characterization of synthesized rohitukine derivatives

$^1$H NMR spectra were run on Bruker Advance DPX 300 MHz in CDCl$_3$. $^{13}$C NMR spectra were recorded at 200 MHz in CDCl$_3$. Chemical shifts are reported as values in ppm relative to CHCl$_3$ (7.26) in CDCl$_3$ and TMS was used as internal standard. ESI mass spectra were recorded on JEOL SX 102/DA-6000. Chromatography was executed with silica gel (100-200 mesh) using mixtures of chloroform and hexane as eluents in varying ratio.

**K-1**  [4-(5,7-dihydroxy-2-methyl-4-oxo-4H-chromen-8-yl)-1-methylpiperidin-3-yl 2-nitro benzenesulfonate]

\[
\text{HO} \quad \text{O} \\
\text{HO} \\
\text{O} \\
\text{NO}_2 \\
\text{N}
\]

Compound K-1 was prepared from rohitukine and 2-nitrobenzene-1-sulfonylchloride using representative procedure to yield pure compound through elution with 80% chloroform in hexane. Yield: 81%; $^1$H NMR: (300 MHz, CDCl$_3$) $\delta$: 12.95 (s, 1H), 8.0-7.50 (m, 4H), 6.50 (s, 1H), 6.28 (s, 1H), 4.96-4.90 (m, 1H), 3.06-2.99 (m, 1H), 2.89-2.81 (m, 3H), 2.55-2.51 (m, 1H), 2.31 (s, 3H), 2.25 (s, 3H), 2.00-192 (m, 1H), 1.47-1.43 (m, 1H); $^{13}$C NMR (200 MHz, CDCl$_3$) $\delta$: 183.81, 166.26, 162.26, 161.86, 161.04, 151.14, 138.38, 137.15, 132.16, 130.60, 128.12, 111.32, 109.82, 106.76, 100.04, 68.02, 56.94, 55.16, 42.17, 27.10, 25.11, 20.78; ESI-MS: m/z 490 (M+1)$^+$

**K-2**  [4-(5, 7-dihydroxy-2-methyl-4-oxo-4H-chromen-8-yl) -1-methylpiperidin-3-yl 4-nitrobenzenesulfonate]

\[
\text{HO} \quad \text{O} \\
\text{HO} \\
\text{O} \\
\text{NO}_2 \\
\text{N}
\]
Compound K-2 was prepared from rohitukine and 4-nitrobenzene-1-sulfonylchloride using representative procedure to afford pure compound through elution with 80% chloroform in hexane. Yield: 72%; \(^1\)H NMR: (300 MHz, CDCl\(_3\)) \(\delta\): 13.16 (s, 1H), 8.19 (d, \(J = 9.2\) Hz, 2H), 7.87 (d, \(J = 9.2\) Hz, 2H), 6.59 (s, 1H), 6.17 (s, 1H), 5.02-4.95 (m, 1H), 3.18-3.11 (m, 1H), 2.80-2.73 (m, 3H), 2.62-2.59 (m, 1H), 2.54 (s, 3H), 2.38 (s, 3H), 1.84-1.79 (m, 1H), 1.76-1.72 (m, 1H); \(^{13}\)C NMR (200 MHz, CDCl\(_3\)) \(\delta\): 182.91, 166.29, 160.49, 160.16, 159.41, 155.14, 152.95, 132.15, 131.98, 125.14, 125.08, 111.25, 110.40, 106.04, 97.24, 67.11, 57.92, 57.22, 43.12, 26.34, 24.82, 20.22; ESI-MS: m/z 490 (M+1)+

K-3  [4-(5,7-dihydroxy-2-methyl-4-oxo-4H-chromen-8-yl) -1-methylpiperidin-3-yl 3-nitrobenzenesulfonate]

Compound K-3 was prepared from rohitukine and 4-nitrobenzene-1-sulfonylchloride using representative procedure to afford pure compound through elution with 90% chloroform in hexane. Yield: 76%; \(^1\)H NMR: (300 MHz, CDCl\(_3\)) \(\delta\): 12.90 (s, 1H), 8.48 (s, 1H), 8.12 (d, \(J = 9\) Hz, 1H), 7.87 (d, \(J = 9\) Hz, 1H), 7.62-7.58 (m,1H), 6.30 (s, 1H), 6.00 (s, 1H), 5.10-5.04 (m, 1H), 3.10-3.03 (m, 1H), 2.72-2.66 (m, 3H), 2.59-2.55 (m, 1H), 2.48 (s, 3H), 2.34 (s, 3H), 1.72-1.68 (m, 1H), 1.60-1.54 (m, 1H); \(^{13}\)C NMR (200 MHz, CDCl\(_3\)) \(\delta\): 184.51, 168.59, 163.24, 162.56,161.84, 152.14, 144.26, 138.82, 132.24, 132.12, 128.24, 112.08, 110.40, 107.11, 99.24, 67.38, 57.44, 56.82, 42.12, 26.84, 26.32, 22.38; ESI-MS: m/z 490 (M+1)+
Compound K-4 was prepared from rohitukine and 4-chlorobenzene-1-sulfonylchloride using representative procedure to afford pure compound through elution with 80% chloroform in hexane. Yield: 68%; $^1$H NMR: (300 MHz, CDCl$_3$) δ: 12.85 (s, 1H), 7.58 (d, $J = 7.8$ Hz, 2H), 7.47 (d, $J = 7.8$ Hz, 2H), 6.44 (s, 1H), 6.25 (s, 1H), 4.89-4.82 (m, 1H), 2.94-2.87 (m, 1H), 2.68-2.64 (m, 3H), 2.62-2.58 (m, 1H), 2.30 (s, 3H), 2.26 (s, 3H), 2.00-192 (m, 1H), 1.78-1.74 (m, 1H); $^{13}$C NMR: (200 MHz, CDCl$_3$) δ: 183.64, 165.96, 162.10, 161.65, 161.02, 150.24, 139.18, 133.25, 132.95, 130.60, 130.24, 110.94, 109.67, 106.24, 99.74, 67.34, 57.98, 56.38, 43.10, 26.10, 25.85, 20.78; ESI-MS: m/z 479 (M+1)$^+$

Compound K-5 was prepared from rohitukine and 2,4-dichlorobenzene-1-sulfonylchloride using representative procedure to afford pure compound at 90% chloroform in hexane as eluent. Yield: 75%; $^1$H NMR (300 MHz, CDCl$_3$): δ: 13.02 (s, 1H), 8.04 (s, 1H), 7.85 (d, $J = 9$ Hz, 1H), 7.78 (d, $J = 9$ Hz, 1H), 6.28 (s, 1H), 6.14 (s, 1H), 5.02-4.97 (m, 1H), 3.18-3.11 (m, 1H), 2.68-2.59 (m, 3H), 2.57-2.54 (m, 1H), 2.41 (s, 3H), 2.34 (s, 3H), 1.87-1.70 (m, 2H); $^{13}$C NMR (200 MHz, CDCl$_3$) δ: 183.42, 167.29, 162.4, 161.6, 158.41, 140.62, 138.23, 135.4, 134.34, 131.19, 129.24, 112.78, 110.65, 107.26, 98.84, 68.57, 57.92, 56.24, 43.47, 27.3, 25.12, 19.98; ESI-MS: m/z 513 (M+1)$^+$
**Dysoxylum binectariferum**

**K-6** [4-(5,7-dihydroxy-2-methyl-4-oxo-4H-chromen-8-yl)-1-methylpiperidin-3-yl 4-methyl benzenesulfonate]

![Chemical Structure of K-6]

Compound K-6 was prepared from rohitukine and 4-methylbenzene-1-sulfonyl chloride using representative procedure to afford pure compound at 80% chloroform in hexane as eluent. Yield: 70%; \(^1\)H NMR (300 MHz, CDCl3) δ: 12.90 (s, 1H), 7.73 (d, \(J = 6\) Hz, 2H), 7.44 (d, \(J = 6\) Hz, 2H), 6.33 (s, 1H), 6.02 (s, 1H), 4.98-4.92 (m, 1H), 3.10-3.06 (m, 1H), 2.96-2.89 (m, 3H), 2.41 (s, 3H), 2.35 (s, 3H), 2.30 (s, 3H), 2.25-2.21 (m, 1H), 1.84-171 (m, 2H); \(^13\)C NMR (200 MHz, CDCl3) δ: 182.94, 166.40, 162.92, 161.46, 158.62, 145.24, 142.25, 131.15, 130.98, 130.24, 130.04, 112.02, 110.90, 107.08, 99.04, 68.10, 58.96, 57.92, 43.12, 25.23, 24.84, 22.56, 20.12; ESI-MS: m/z 459 (M+1)^+

**K-7** [4-(5,7-dihydroxy-2-methyl-4-oxo-4H-chromen-8-yl)-1-methylpiperidin-3-yl 2,4,6-trimethylbenzenesulfonate]

![Chemical Structure of K-7]

Compound K-7 was prepared from rohitukine and 2,4,6-trimethylbenzene-1-sulfonyl chloride using representative procedure to afford pure compound at 90% chloroform in hexane as eluent. Yield: 68%; mp: \(^1\)H NMR (300 MHz, CDCl3) δ: 13.06, 7.10 (s, 2H), 6.29 (s, 1H), 6.02 (s, 1H), 4.96-4.91 (m, 1H), 3.16-3.10 (m, 1H), 2.81-2.76 (m, 3H), 2.56 (s, 6H), 2.46-2.42 (m, 1H), 2.40 (s, 3H), 2.35 (s, 3H), 2.29 (s, 3H), 1.90-1.82 (m, 2H); \(^13\)C NMR (200 MHz, CDCl3) δ: 184.12, 166.72, 163.24, 163.12, 161.24, 142.12, 138.84, 137.23, 137.19, 130.42, 130.40,
**Dysoxylum binectariferum**

112.78, 110.65, 105.82, 96.48, 67.95, 63.22, 5812, 56.82, 44.38, 24.42, 23.24, 23.21, 22.12, 19.44; ESI-MS: m/z 487 (M+1)^+

**K-8**  [4-(5,7-dihydroxy-2-methyl-4-oxo-4H-chromen-8-yl)-1-methylpiperidin-3-yl 2,4,6-triisopropylbenzenesulfonate]

![Chemical Structure of K-8](image)

Compound K-8 was prepared from rohitukine and 2,4,6-triisopropylbenzene-1-sulfonyl chloride using representative procedure to afford pure compound at 80% chloroform in hexane as eluent. Yield: 77%; ^1^H NMR (300 MHz, CDCl3) δ: 12.98 (s, 1H), 7.60 (s, 2H), 6.20 (s, 1H), 6.04 (s, 1H), 5.02-4.96 (m, 1H), 3.21-3.14 (m, 2H) 2.82-2.64 (m, 6H), 2.36 (s, 3H), 2.29 (s, 3H), 1.84-1.75 (m, 2H) 1.30 (s, 6H), 1.27 (s, 6H), 1.24 (s, 6H), ^1^3^C NMR (200 MHz, CDCl3) δ: 184.12, 167.82, 162.44, 162.16, 160.10, 156.12, 156.08, 154.15, 154.16, 126.24, 124.82, 124.77, 111.78, 110.65, 106.12, 99.48, 67.15, 59.42, 57.22, 43.12, 36.46, 36.44, 35.82, 26.12, 25.90, 24.80, 24.77, 24.42, 24.40, 24.04, 24.02, 21.24; ESI-MS: m/z 571 (M+1)^+

**K-9**  [4-(5,7-dihydroxy-2-methyl-4-oxo-4H-chromen-8-yl) -1-methylpiperidin-3-yl 4-methoxy benzenesulfonate]

![Chemical Structure of K-9](image)

Compound K-9 was prepared from rohitukine and 4-methoxybenzene-1-sulfonyl chloride using representative procedure to afford pure compound at 80% chloroform in hexane as eluent. Yield: 74%; ^1^H NMR (300 MHz, CDCl3) δ: 12.98 (s, 1H), 7.42 (d, J = 8.52 Hz, 2H), 6.96 (d, J = 8.52 Hz, 2H ), 6.23 (s, 1H), 6.05 (s, 1H), 4.97-4.91 (m, 1H), 3.37 (s, 3H), 3.18-3.10 (m, 1H), 2.74-
Dysoxylum binectariferum

2.66 (m, 3H), 2.41 (s, 3H), 2.35 (s, 3H), 2.29-2.25 (m, 1H), 2.02-1.84 (m, 2H); $^{13}$C NMR (200 MHz, CDCl$_3$) δ: 185.22, 168.48, 165.94, 160.59, 160.16, 159.12, 141.67, 135.12, 135.12, 117.28, 117.28, 112.04, 110.02, 106.12, 99.14, 67.17, 58.74, 56.34, 55.40, 43.87, 25.74, 25.24, 21.28; ESI-MS: m/z 475 (M+1)$^+$

**K-10** [4-(5,7-dihydroxy-2-methyl-4-oxo-4H-chromen-8-yl)-1-methylpiperidin-3-yl naphthalene-1-sulfonate]

![Compound K-10](image)

Compound K-10 was prepared from rohitukine and 4 naphthalene-1-sulfonyl chloride through representative procedure to afford pure compound at 90% chloroform in hexane as eluent. Yield: 80%; $^1$H NMR (300 MHz, CDCl$_3$) δ: 12.90 (s, 1H), 8.70 (d, J=8.49, 1H), 8.13 (d, J=7.71, 1H ), 8.11 (d, J=6.96, 1H ), 7.94 (d, J=8.01, 1H) 7.71, (t, J=7.92), 7.63-7.49 (m, 2H) 6.10 (s), 5.98 (s), 4.95-4.90 (m1H, ), 3.07-3.01 (m, 1H), 2.74-2.68 (m, 3H), 2.57-2.52 (m, 1H), 2.32 (s, 3H), 2.26 (s, 3H) 2.12-1.96 (m, 2H); $^{13}$C NMR (200 MHz, CDCl$_3$) δ:183.22, 167.35, 163.23, 163.02, 160.46, 144.74, 135.91, 132.82, 130.80, 130.14, 128.82, 128.71, 126.90, 126.74, 125.51, 111.72, 110.10, 106.86, 98.94,69.06, 58.22, 55.12, 42.32, 25.83, 25.24, 20.54; ESI-MS: m/z 495 (M+1)$^+$

**K-11** [4-(5,7-dihydroxy-2-methyl-4-oxo-4H-chromen-8-yl)-1-methylpiperidin-3-yl thiophene-2-sulfonate]

![Compound K-11](image)

Compound K-11 was prepared from rohitukine and thiophene-2-sulfonyl chloride through representative procedure to afford pure compound at 90% chloroform in hexane as eluent. Yield:
Dysoxylum binectariferum

70%; $^1$H NMR: (300 MHz, CDCl$_3$) $\delta$: 12.92 (s, 1H), 7.62-7.38 (m, 3H), 6.32 (s, 1H), 6.04 (s, 1H), 5.14-5.08 (m, 1H), 3.12-3.05 (m, 1H), 2.70-2.63 (m, 1H), 2.56-2.52 (m, 1H), 2.45 (s, 3H), 2.31 (s, 3H), 2.17-2.16 (m, 1H), 1.75-1.51 (m, 1H); $^{13}$C NMR (200 MHz, CDCl$_3$) $\delta$: 184.51, 168.59, 163.24, 162.56, 161.84, 129.12, 128.20, 128.02, 124.24, 112.08, 110.40, 107.11, 99.24, 67.98, 58.44, 56.82, 42.12, 25.84, 25.64, 22.38; ESI-MS: m/z 451 (M+1)$^+$

**BIOLOGICAL ACTIVITY: ANTICANCER**

Cancer is a multifactorial disease with excessive and robust biological networks and is a major public health burden of present era. It is thought to be caused by the interaction between genetic susceptibility and environmental factors. In addition to developed countries developing countries are also being increasingly afflicted with cancer, due to increased life expectancy and advanced pattern of socio-cultural life dominated by western medicines. It requires treatment with chemotherapeutic agents with specific targets for example, the cell cycle. This has led to a search for materials that have specific targets in cancerous cells that control the cell cycle. In fact compounds that target multiple intracellular components and distinct molecular mechanisms may be preferable and considered more promising.

Prostate cancer in men and breast cancer in women are the most commonly occurring cancers in western world. Treatment with chemotherapeutic agents in both prostate and breast cancers can prolong the life span but current achievements in cancer therapy fail to attain sure shot treatment. Tamoxifen, an ER agonist acting through ER receptor is the most effective, selective estrogen receptor modulator (SERM) with breast cancer specific activity. It is the drug of choice in inhibiting estrogen action in ER $^+$ve breast cancer but is effective only against ER positive tumors. Similarly, the docetaxel is the only drug which offers survival benefits in advanced stages of prostate cancer. All the current cancer treatments are very expensive and have significant side effects. In this regard, there is considerable interest in the design and development of novel molecules with higher efficacy, selective anticancer activity and human body tolerability. Accumulating evidence has shown that natural products and their semi-synthetic derivatives remain one of the major sources of potential anti-cancer agents with nearly 50% of the new chemical entities launched in the market [44]. Rohitukine is one such molecule which has anticancer activity against both breast and prostate cancer cell lines, our aim is to prepare new semisynthetic analogs of rohitukine in order to obtain new anticancer molecules. Sulphonyl
derivatives (K1- K11) have been synthesized and evaluated for their cytotoxic potential at different doses in breast (MCF-7 and MDA-MB-231) and prostrate (PC-3 and DU-145) cancer cell lines to establish their anticancer potential, compounds K-6, K-8 and K-10 exhibited exciting results in MCF-7 cell line so they were further assessed for various parameters.

**Materials and Methods**

Dichlorodihydro-fluorescein diacetate dye (DCF-DA), 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), propidium iodide (PI) and rhodamine 123, were purchased from Sigma Chemical Company (St. Louis, CA USA). Antibodies specific for Bad, caspase-3, p53, Bcl-2, Bcl-xL and and β-actin were procured from Cell signaling Technology, Inc., (Danvers, MA USA). The anti-rabbit horseradish-peroxidase conjugate secondary antibodies were obtained from Bangalore Genei (Bangalore, India).

**Cell culture.**

Breast cancer cell lines (MCF-7, MDA-MB-231) and Prostate cancer cell lines (DU-145, PC-3) were obtained from American Type Culture Collection (Manassas, VA, USA). All the culture media were obtained from Invitrogen (Melbourne, VIC, Australia). Cells were cultured in Dulbecco’s Modified Eagle Media (DMEM) supplemented with 10% heat inactivated fetal bovine serum, 100 μg/mL penicillin, streptomycin (Gibco Lifetech, Karlsruhe, Germany). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO2 inside a CO2 incubator.

**The compounds K-1 to K-11 were evaluated for the following parameters**

**Anti-proliferative activity**

The antiproliferative activity of compounds K-1 to K-10 was determined through MTT assay [45]. All the compounds were evaluated for cytotoxic potential in Breast cancer cell lines (MCF-7 and MDA-MB-231) and Prostate cancer cell lines (PC-3 and DU-145). 1×10⁴ cells/well were seeded in 96-well microculture plates in 100 μL DMEM media supplemented with 10% FBS in each well and incubated in a CO₂ incubator for 24 h at 37 °C. Compounds were diluted to the desired concentrations and added in the culture medium in the wells with respect to the vehicle control. Media were removed after 48 h of incubation and 100 μL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (5 mg/mL) was added to each well and plates were
further incubated in CO$_2$ incubator for 4 h. Supernatant was removed carefully from each well, formazan crystals were dissolved in 100 µL of DMSO and the absorbance were recorded at 540 nm wavelength.

**Analysis of mitochondrial membrane potential (MMP)**

The MMP was measured by the uptake of unique fluorescent cationic dye, JC-1 (excitation at 488 nm and emission at 525 nm), to signal the loss of MMP [46]. This fluorescent probe exists as a green fluorescent monomer (emission 527 nm) at low MMP. Mitochondrial depolarization is indicated by an increase in green fluorescence (FL-1). The MCF-7 cells (0.2 × 10$^6$ cells) were seeded in a 6-well plate and exposed to compounds at varying concentrations for 36 h. After that the cells were washed and finally harvested in chilled PBS containing JC-1 (1 µM). The samples were incubated at 37°C for 30 min in the dark, washed twice with chilled PBS and finally resuspended in 200 µL PBS. Mitochondrial permeability transition was subsequently quantified on FACS.

**Measurement of intracellular ROS**

For analysis of intracellular ROS, the oxidation- sensitive probe DCF-DA was used to analyze the net intracellular generation of ROS through flowcytometry [47]. After 36 h of incubation in the absence or presence of compounds at varying concentrations, cells were detached by trypsinization and the cellular fluorescence intensity was measured after 30 min incubation with (DCFDA 10 µM) using flow cytometer. Propidium iodide (0.005%) was used to detect dead cells. For each analysis, 10,000 events were recorded.

**Caspase assay**

The homogeneous caspase assay kit (Roche, Germany) that includes caspases 2, 3, 6, 7, 8, 9 and 10 was used to measure caspase activities followed by induction of apoptosis according to manufacturer’s instructions. Camptothecin treated U937 cells were used as positive control. MCF-7 cells were cultured under serum-deprived conditions for 4 h and exposed to compounds at varying concentrations for 36 h. Cells were then incubated with DEVD-rhodamine- 110 (tetrapeptide sequence ‘aspartic acid–glutamic acid–valine–aspartic acid’ recognized by caspases). Upon cleavage of the rhodamine substrate by activated caspases, fluorescence from the released rhodamine-110 was measured.
Assessment of apoptosis by flow cytometry

The percentage of cells undergoing apoptosis was determined using Annexin V FITC Assay kit (BD Biosciences). Annexin V is a Ca\(^{2+}\)-dependent phospholipid binding protein which has high affinity for phosphatidylserine (PS), and fluochrome-labeled Annexin V is used for the detection of exposed phosphatidylserine and Propidium Iodide (PI) is used for the differentiation from necrotic cells using flow cytometry. The MCF-7 cells were cultured and treated with compounds at varying concentrations for 36 hr. Subsequently cells were washed twice with PBS, and centrifuged at 1200 rpm for 5 min. Afterwards cell pellet was treated with 5 µl Annexin V and 5 µl of PI and incubated for 15min. in dark. Finally 200 µl of binding buffer was added to the cell pellet and analyzed by flow cytometry (BD Biosciences).

Western Blot Analysis

Cell pellets were lysed in RIPA buffer containing 50 mM Tris pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP-40, 1 mM DTT, 1 mM NaF, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride PMSF, (Sigma), and 1% protease inhibitors cocktail (Merk). Protein concentration was determined using the Lowry method (Bio-Rad). Equivalent amount (20µg) of total protein was loaded onto 12% SDS-PAGE [48]. The gel was transferred to nitrocellulose membrane using an electro-blotting apparatus (Bio-Rad, Richmond, CA). Membranes were blocked with 5% nonfat milk in TBS-T buffer [10 mM Tris HCl (pH 7.5), 150 mM NaCl, 0.05% Tween 20] and incubated with primary antibodies at 4\(^{\circ}\)C, overnight. Blots were then washed with TBS-T and incubated with horseradish peroxidase-conjugated secondary anti-rabbit antibody. ß-Actin was used as internal control. The membranes were developed with ECL reaction and analyzed using LAS- 3000 Luminescent Image Analyzer (FujiFilm, Tokyo, Japan). ImageGauge Ver. 3.0 software was used to calculate the changes in protein expression.

Results

We embark to synthesize novel semisynthetic derivatives of the lead molecule rohitukine. Compounds K1-K10 were evaluated for antiproliferative activity against MCF-7, MDA-MB-231, PC3 and DU-145 cell lines alongside Doxorubicine, standard drug. The cytotoxic activities for these compounds are presented in Table 2.3. Compounds K-6, K-8 and K-10 showed promising activity.
Table 2.3: IC\textsubscript{50} Values of K1-K10 in different Cancer Cell Lines

<table>
<thead>
<tr>
<th>S. N.</th>
<th>(IC50 values in μM)</th>
<th>Compounds</th>
<th>MCF-7</th>
<th>MDA-MB-231</th>
<th>PC-3</th>
<th>DU-145</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>K-1</td>
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<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;50</td>
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</tr>
<tr>
<td>2</td>
<td>K-2</td>
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<td>&gt;50</td>
<td>42.2</td>
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</tr>
<tr>
<td>3</td>
<td>K-3</td>
<td>46.3</td>
<td>47.4</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>K-4</td>
<td>44.7</td>
<td>&gt;50</td>
<td>45.9</td>
<td>&gt;50</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>K-5</td>
<td>44.3</td>
<td>&gt;50</td>
<td>40.2</td>
<td>&gt;50</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>K-6</td>
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<td>35.0</td>
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</tr>
<tr>
<td>7</td>
<td>K-7</td>
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<td>&gt;50</td>
<td>42.2</td>
<td>&gt;50</td>
<td></td>
</tr>
<tr>
<td>8</td>
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<td>22.8</td>
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<td>9</td>
<td>K-9</td>
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<td>47.5</td>
<td>&gt;50</td>
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</tr>
<tr>
<td>10</td>
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<td>Doxorubicin</td>
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<td>22.2</td>
<td>14.2</td>
<td>20.4</td>
<td></td>
</tr>
</tbody>
</table>

Compounds K-6, K-8 and K-10 exhibited appreciable antiproliferative activity in all the four cancer cell lines showing minimum IC\textsubscript{50} values against MCF-7. Each experiment was repeated thrice and standard deviations were derived from three independent experiments. IC\textsubscript{50} of K-6, K-8 and K-10 are 17.5, 17.0 and 19.0 μM respectively, thus these semisynthetic analogs of...
Rohitukine have been found to be effective against breast and prostate cancer cell lines and this is the rationale to further evaluate various parameters to determine the mechanism of action of these derivatives. In order to explore the mechanism of inhibition of cell growth, most active compounds K-6, K-8 and K-10 were evaluated in MCF-7 cells for the detection of pathway. The compounds K-6, K-8 and K-10 showed significant decrease in mitochondrial membrane potential in a dose dependent manner which is reflected by decrease in mean fluorescence with respect to control (Figure 2.3). (MCF -7 displayed basal fluorescence indicating functional mitochondria).

Figure 2.1: Assessment of IC$_{50}$ value of compound K-6 in MCF-7 cells
Figure 2.2: Assessment of IC<sub>50</sub> values of compound K-8 and K-10 in MCF-7 cells
Figure 2.3: Effect of compounds K-6, K-8 and K-10 on loss of MMP. Trypsinized cells were incubated with the fluorescent cationic dye, JC-1 (excitation at 488 nm and emission at 525 nm) (1 µM) for 30 min at 37°C in the dark, washed twice with chilled PBS and finally resuspended in 200µL PBS. The mitochondrial permeability transition was quantified on FACS. Data shown are the mean ± SE of one of three similar experiments each performed in triplicate *P < 0.05, **P < 0.01, ***P < 0.001 (as compared to control cells).
Treatment of MCF-7 with compounds K-6, K-8 and K-10 showed significant increase in intracellular ROS in a dose dependent manner which is reflected by increase in mean DCFDA fluorescence with respect to control (Figure 2.4).

**Figure 2.4:** Effect of compounds K-6, K-8 and K-10 on ROS generation. Cells were exposed for 36h at different concentrations of compounds, washed twice with chilled PBS, followed by incubating the cells with 10μM DCFDA fluorophore for 30 min at 37°C in the dark, washed twice with chilled PBS and trypsinized. Finally, the stained cells were analyzed through flow cytometry. Data shown are the mean ± SE of one of three similar experiments each performed in triplicate *P < 0.05, **P < 0.01, ***P < 0.001 (as compared to control cells).
Figure 2.5: Effect of compounds K-6, K-8 and K-10 on induction of caspase in MCF-7. Cells were treated with compounds for 36 h at various concentrations, and incubated with DEVD-rhodamine-110. Fluorescence from the activated caspases-mediated release of rhodamine-110 was measured at 560 nm. Data presented are mean ± SE of one of three similar experiments each performed in triplicate *P < 0.05, **P < 0.01, ***P < 0.001 (as compared to control cells).
Figure 2.6: Assessment of apoptosis - percentage of apoptotic cells of MCF-7 cells followed by treatment with compounds K-6, K-8 and K-10 at 15µM, 18µM, 21 µM and 24µM concentrations. Data presented are mean ± SE of one of three similar experiments each performed in triplicate **P < 0.01, ***P < 0.001 (as compared to control cells).

Caspases are cysteine proteases which constitute cascade that get activated sequentially during the caspase dependent apoptosis and thus caspase activation is used as a measure to assess the pathway. In order to determine that whether the apoptosis induced by compounds K-6, K-8 and K-10 was caspase dependent, these compounds were evaluated in MCF-7 cells for caspase activity.
Treatment of MCF-7 cells with different doses of compounds K-6, K-8 and K-10 for 24 h and later incubation with DEVD-rhodamine-110, significantly activated caspases and cleaved rhodamine substrate (Figure 2.5). Fluorescence was measured at 560 nm. All the three compounds significantly induced basal caspase level indicating that apoptosis is caspase dependent.

Percentage of cells undergoing apoptosis was determined using flow cytometry. Treatment of MCF-7 cells with compounds K-6, K-8 and K-10 showed induction of apoptosis in a dose dependent manner (Figure 2.6).

**Figure 2.7:** Effect of most active compounds K-6, K-8 and K-10 on expression of different apoptotic factors.
Analysis of western blot indicated that treatment with compounds K-6, K-8 and K-10 upregulated the pro apoptotic proteins Bax, p53 and cleavage of caspase -3 while downregulated the anti apoptotic protein Bcl-2 which is indicative of the mechanistic pathway by which the active compounds mediated the apoptosis in MCF-7.

**Discussion**

*Dysoxylum binectariferum* stem bark as well as its major active constituent rohitukine possesses diverse biological activities including anti-inflammatory, immunomodulatory, anti leishmanial and anti cancer activities. However, for the first time its Sulphonyl derivatives have been evaluated against prostate and breast cancer cell lines, compounds K-6, K-8 and K-10 have shown significant results against prostate and breast cancer cell lines. It is shown to impart its anti-cancer property by induction of apoptosis. Detailed study revealed the molecular mechanism involved in apoptosis caused by these semisynthetic derivatives. After assessing the results of various parameters like mitochondrial membrane potential, ROS, caspase induction and western blot, it is evident that apoptosis is caspase mediated.

Apoptosis is triggered by sequence of events such as generation of ROS [49], the primary site for which is mitochondrial membrane. Generation of ROS causes decrease in MMP, it is also known that elevated ratio of Bax and Bcl-2 is inversely proportional to MMP and can cause its collapse [50, 51]. In normal condition, Bcl-2 induce the inactivation of Bax through forming a heterodimer but in the presence of pro-apoptotic signals, Bax migrate to mitochondria and facilitates the release of cytochrome c from it and promote the formation of apoptosome with Apaf1 which in turn activates caspases leading to the apoptosis [52, 53]. Activation of caspases interferes with normal functions of PARP which is to repair damaged DNA. Upregulation of pro-apoptotic proteins like Bax, p53 and down regulation of anti-apoptotic proteins like Bcl-2 gives insight into the mechanism of action followed by compounds K-6, K-8 and K-10 to induce the apoptosis. The synthesis of these semisynthetic derivatives from rohitukine have serve their purpose to an extent and taking lead from them, other derivatives with better potential as well as novel mechanism of action can be derived to combat cancer.
BIOLOGICAL ACTIVITY: ANTIADIPOGENIC

Obesity and associated disorders now crossed epidemic state and entered pandemic proportions [54]. Obese people are at the risk of decreased life expectancy and contribute to increased national health burden in terms of budget and productive manpower loss. Although factors responsible for obesity are not completely understood, increase in lipid content of fat storing cells i.e. adipocytes is considered relevant. These cells increase glucose and fatty acid uptake to store it in form of triglyceride during nutritional excess and serve as energy reservoir. In nutritional deprivation conditions, it provides energy in form of fatty acid and glycerol and thus involved in both catabolic and anabolic processes. Apart from this, by virtue of its capacity to secret various adipokines and cytokines, adipocytes gained status of an important endocrine tissue [55]. New adipocytes are constantly made in order to replace old one and in this process approximately 50% sub-cutaneous adipocytes are replaced every 8 years. Several natural products are known to affect adipogenic differentiation at various life cycle stages of adipocyte [56-60]. These effects are exerted either by induction of apoptosis, inhibition of adipogenesis and/or by stimulating lipolysis. In present study, we investigated effect of rohitukine on adipocyte differentiation in 3T3-L1 and C3H10T1/2 cells under influence of hormonal inducers. We measured effects in terms of lipid accumulation, gene and protein level expression for various adipogenesis associated marker proteins. Our studies conclude rohitukine affects early phase of adipogenic differentiation by inhibiting AKT-mTOR phosphorylation, causes S-phase cell cycle arrest during mitotic clonal expansion (MCE). Rohitukine, at later time points, downregulates expression of PPAR-γ and C/EBP-α and leads to inhibition of adipogenesis.

Materials and Methods

Differentiation of 3T3-L1 and C3H10T1/2 adipocytes

3T3-L1 and C3H10T1/2 cell lines were purchased from the American Type Culture Collection. Cells were cultured in a humidified atmosphere at 37°C and 5% CO₂ in Dulbecco’s modified Eagle’s medium containing 10% (v/v) heat-inactivated fetal bovine serum and Penicillin-streptomycin antibiotics (cDMEM). For adipogenic induction cells were seeded in 24-multi-well plates. Two day post confluence, culture media was replaced with differentiation medium (MDI) (medium containing Insulin 5 µg/ml, IBMX 0.5 mM and Dexamethasone 250 nM). This media was replaced after 48 hr with medium containing 5 µg/ml Insulin. This medium was replaced...
after 48 hours and cells were maintained in cDMEM. Lipid droplet started appearing from day 4th and >90% cells show lipid globules after 6-8 days after induction. To study the effect of rohitukine on adipogenic differentiation, cells were differentiated at given concentrations and/or time exposed to rohitukine as indicated in figures.

**Oil Red-O (ORO) staining**

Differentiated 3T3-L1 (with or without compound) adipocytes were rinsed in phosphate buffered saline (PBS) (pH 7.4). The adipocytes lipid globules were stained with ORO (0.36% in 60% Isopropanol) for 20 min. Unstained Oil Red-O was removed by rinsing wells twice with phosphate buffered saline. After complete removal of PBS, dye was extracted from cells using 100% Isopropanol and measured absorbance at 492 nm.

**Western blotting**

Cells were lysed in ice-cold mammalian lysis buffer containing 0.5M EDTA, protease inhibitor (Amresco) and phosphatase inhibitor Phos-STOP (Roche). Protein concentrations were measured by Bicin-cholinic acid method (Sigma). Protein lysates were denatured by heating at 65 °C for 10 minutes in Laemmli sample buffer supplemented with 10% β-mercaptoethanol and equal quantity of protein was resolved by 8 to12% SDS-PAGE and transferred to Nitrocellulose paper by electro-transfer overnight. The membranes were blocked with 5% skimmed milk (Sigma) in tris-buffered saline containing 0.05% Tween-20 (TBS-T). After washing with TBS-T, the membranes were incubated with target protein specific antibodies for 14hrs at 4°C, followed by incubation with appropriate HRP-conjugated secondary antibodies for 1 hour. The target proteins were detected using Immobiline western Chemiluminescence detector (Millipore) according to the manufacturer’s protocol. For equal loading and normalization purpose, β-actin was used as internal loading control.

**Real Time PCR**

Total RNA was isolated from 3T3-L1 cells using TRIZOL reagent (Invitrogen CA, USA). First strand cDNA synthesis was performed using Megascript reverse transcriptase kit (Applied Biosystems) and subsequently used for quantitative real time PCR analysis on Light Cycler 480 using SYBR Green master mix (Roche Diagnostics). Statistical analysis of the quantitative real time PCR obtained using the (2-ΔΔCt) method, which calculates the relative changes in gene
expression of the target, normalized to an endogenous reference (GAPDH) and relative to a calibrator that serves as the control group.

**Cell cycle analysis using flow Cytometry**

Two day postconfluent 3T3-L1 preadipocytes were incubated in differentiation medium (MDI) with or without rohitukine at different concentrations. The cells were harvested after 24 hours, washed and re-suspended in PBS. Then, cells were fixed in 70% ice-cold ethanol. Pelleted cells were suspended in propidium iodide for 30 minutes at room temperature. At least 10000 events were acquired per sample on flow cytometer (BD, FACSCalibur). Analysis was performed using Modfit software to determine the relative amount of cells in G1, S and G2/M.

**Pharmacokinetic studies- Bioavailability estimation**

*In-vivo* pharmacokinetics study was performed in male Golden Syrian hamster (n=3, weight range 120.0 ± 10 gm). Oral and intra-venous (i.v.) pharmacokinetics of rohitukine was carried out at 50 mg/kg and 5 mg/kg respectively for bioavailability estimation. Animals were fasted overnight prior to dosing but allowed free access to water. Blood samples were collected from the retro orbital plexus into micro-centrifuge tubes containing heparin (20 IU/ml) as anti-coagulant at 0.25-48.0 hr post oral dosing and 0.08 – 24 hr post i.v. dosing. Plasma was harvested by centrifuging the blood samples at 2000 × g for 5 min. The plasma samples (300 µL) were extracted by solid phase extraction (SPE) method using 1 cc, C18 (DSC18, Supelco) cartridge. Cartridges were conditioned with methanol and 10 mM Sodium Acetate buffer pH 5.5. Plasma samples (300 µL) with internal standard (IS) phenacetin (5µg/ml) were loaded into the cartridges. Then cartridges were washed with 10mM Sodium Acetate buffer pH 5.5, eluted with 2 ml of methanol and dried under vacuum. The dry residues reconstituted in 100 µl methanol and 50 µl supernatant was taken for HPLC analysis. Rohitukine as well as IS was resolved on Phenomex C18 column (4.6 ×250 mm, particle size 5 µm) with isocratic mobile phase consisting of methanol : 10mM sodium acetate buffer aqueous formic acid (pH 5.5) 62:38, at a flow rate of 1.0 ml/min. The absorption wavelength was set to 257 nm and 240 nm for rohitukine and IS, respectively. Pharmacokinetic parameters such as area under the plasma concentration–time curve were analyzed with standard non-compartmental method with WinNonLin software program (version 4.1, Pharsight Corporation, CA).
Statistical analysis

Data were expressed as mean+SD Students t-test was used for comparisons of measured parameters. A probability value of P<0.05 and/or P<0.01 was used as measure of statistical significance. Data was analyzed on Graph Pad Prism (Version3.00, Graph pad Software Inc. San Diego, CA, USA).

Results

Rohitukine inhibits adipogenesis

Varying concentrations of rohitukine (0-20 µM) was added to MDI during differentiation. Microscopic observation showed rohitukine decreased lipid droplet accumulation in differentiated cells in concentration dependent manner (Figure 2.8 a). Absorbance of extracted ORO accumulated in lipid droplets confirms that rohitukine inhibits adipogenesis significantly at 5µM concentration and more than 80% lipid accumulation is inhibited at 20µM concentration (Figure 2.8 b). At this concentration, rohitukine found to be nontoxic in 3T3-L1 cells using MTT assay. MCE blockade was also confirmed by [3H]-thymidine uptake assay. Rohitukine significantly inhibited MDI induced [3H]-thymidine incorporation after 48 hours exposure (Figure 2.10 d) at 20µM concentration. It was observed that even initial 48 hour exposure of rohitukine achieved very significant reduction in lipid droplet accumulation and adipogenesis (Figure 2.8 e). Consistent with these results, we observed that at 20 µM concentration, gene level expression of adipogenesis associated genes viz. PPAR-γ, FABP-4 (ap2), SREBP-1c, FAS and LPL were significantly reduced (Figure 2.9 a), while a decreasing trend was observed in case of C/EBP-α in real time PCR analysis. The anti-adipogenic transcription factors GATA2 and Wnt3a gene expression were increased significantly 30 and 50 fold in presence of rohitukine (Figure 2.9 d). This was also supported by fact that protein level expression of PPARγ, C/EBPα and glut-4 expression was found to be suppressed significantly as compared to control during late phase of adipogenesis. In initial 48 hours rohitukine shows increased expression of C/EBP-α compared to only MDI treated cells.
Figure 2.8: Effects of rohitukine on MDI-induced adipogenesis in 3T3-L1 and C3H10T1/T2 preadipocytes, (a,b,c): dose dependent, (d,e,f): time dependent. Data are expressed as means±s.d. Significant differences between the group treated with MDI only and the group treated with MDI and rohitukine (***P>0.01 and **P>0.001, respectively).
We further evaluated anti-adipogenic effect of rohitukine in C3H10T1/2 cells, a mouse mesenchymal stromal cell line. Rohitukine addition to MDI inhibits adipogenesis in concentration and time dependent manner. This was confirmed qualitatively by microscopy (Figure 2.8 a and d) and quantitatively by ORO absorbance (Figure 2.8 c and f). Collectively these results indicate that, rohitukine possesses an anti-adipogenic potential in-vitro without any cyto-toxic effect and early 48 hour exposure is sufficient to suppress adipogenesis significantly.

**Figure 2.9:** (a, d): Effect of rohitukine on mRNA expression of adipogenic transcription factors C/EBPα, PPARγ SREBP-1c, LPL, aP2 and FAS as well as anti-adipogenic transcription factors GATA2 and Wnt3a. The results were verified by three of the experiments, each of which was conducted in triplicate means±s.d. Significant difference between the control and rohitukine treated groups (*P>0.05, **P>0.01, ***P>0.001). (b,c): Effect of rohitukine on expression of GLUT-4, PPARγ and C/EBPα as well as AKT/mTOR phosphorylation in 3T3-L1 adipocytes. Data are representative of three independent experiments that showed the same tendency.

**Rohitukine arrests mitotic clonal expansion in S- phase**

When MDI is added to growth arrested confluent cells, it imposes cells to enter into 2-3 rounds of cell cycles i.e MCE which is pre-requisite for adipogenesis. Addition of rohitukine to MDI
perturbed MCE at 20μM concentrations of rohitukine where maximum suppression of adipogenesis is observed, 41.15% cells were in S-phase compared to that of only 19.28% cells in MDI alone treated cells (Figure 2.10 b). This highly significant increase demonstrates that cells either get delayed entry into S-phase or get arrested in S-phase.

MCE arrest was further evidenced in immuno-blot expression analysis of cell cycle regulatory proteins performed at 16h and 24h. time-points. Rohitukine causes reduction of CDK2, CDK4, Cyclin A and E. These proteins required to accomplish MCE in response to MDI induction. Furthermore addition of rohitukine in MDI causes rescue of degradation of P27, and reduced expression of CEBPβ, that can be attributed to inhibition of CDK and MCE arrest respectively.

**Rohitukine oral bioavailability studies**

The therapeutic response of a drug is correlated with its availability in systemic circulation. Oral bioavailability (50 mg/kg) was carried out in Syrian Golden hamsters. The studies showed that rohitukine is readily bioavailable and approximately 25.7 % rohitukine get absorbed in systemic circulation when administered orally (Table 2.4). Rohitukine is rapidly absorbed from gastrointestinal tract after oral administration and it could be detected in plasma within 15 min post oral dose. Rohitukine remains in circulation for more than 24 hours.

**Table 2.4: Pharmacokinetic profile of rohitukine after oral (50 mg/kg) and I.V. (5 mg/kg) administration (n=3)**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Estimates (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Oral (50 mg/kg)</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (µg / ml)</td>
<td>6.62 ± 0.66</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; (hr)</td>
<td>0.25</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-∞&lt;/sub&gt; (hr*µg / ml)</td>
<td>10.66 ± 2.34</td>
</tr>
<tr>
<td>MRT (hr)</td>
<td>6.64 ± 1.39</td>
</tr>
<tr>
<td>F&lt;sub&gt;abs&lt;/sub&gt; (%)</td>
<td>25.7</td>
</tr>
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</table>

Abbreviations: C<sub>max</sub>: Maximum concentration, T<sub>max</sub>: Time of Maximum concentration, AUC: area under the curve from 0 to ∞ hr, MRT: mean residence time
Figure 2.10: (a,b). Effects of rohitukine on MDI-induced cell cycle progression in 3T3-L1 preadipocytes, Rohitukine inhibited MDI-induced cell cycle progression in G2/M phase. The data are representative of more than three independent experiments with similar results. The population of cells in each stage of the cell cycle was quantified and showed the dose dependent arrest in G2/M phase. (c). Effects of rohitukine on expression of Cyclin-D, Cyclin-E, CDK-4, CDK-2, p-27 and CEBP-β. (d) Effects of rohitukine on [3H]-thymidine incorporation in 3T3-L1 preadipocytes.
Discussion
Adipose tissue is central to lipid and glucose metabolism. Pre-adipocyte fibroblast clonal cell line 3T3-L1 is considered as an excellent model system to identify molecules affecting adipogenesis process and lipid accumulation capacities. Many phenolic and flavanoid compounds are able to inhibit lipogenesis and adipogenesis.

In this study rohitukine showed anti-adipogenic activity in more than one cell models of *in vitro* adipogenesis. On subsequent investigations, rohitukine exhibited concentration dependent as well as exposure time dependent decrease in ORO accumulation during 3T3-L1 adipogenic differentiation. Anti-adipogenic potential of rohitukine was also confirmed by mouse mesenchymal stromal cell line C3H10T1/2. Inhibitory effect was prominent in early phase of differentiation, possibly due to affecting S-phase arrest in mitotic clonal expansion. Adipogenesis is orchestrated by various genes and proteins. In general, PPARγ is considered as master regulator of adipogenic programming. PPARγ along-with C/EBPα leads to various protein expressions correlating to increasing adipogenesis. C/EBPα and PPARγ cross regulate each other through positive feedback loop and trans-activate downstream target genes such as fatty acid binding protein-4( aP2), Lipoprotein lipase (LPL), Sterol regulatory element binding protein (SREBP-1c) and Fatty acid binding protein 1c [61]. GATA2 and GATA3 negatively regulate PPAR-γ and C/EBP-α expression and thus responsible for decreased adipogenesis [62]. Similarly Wnt pathway is also negatively regulate adipogenic induction. It is also reported that early phase increased expression of C/EBPα contributes negatively to adipogenesis while late phase expression contributes to adipogenesis. When 20µM rohitukine was co-incubated during adipogenesis programming, it shows decreased expression levels of PPAR-γ and Glut4 in late phase expression profile (Figure 2.9 b). C/EBPα is overexpressed on day 2 compared to vehicle control while late phase expression is moderately decreased. Expression of all marker proteins *viz.* LPL, aP2, SREBP-1c, FAS and PPAR-γ reduced significantly when estimated by real time PCR after 8 days of adipogenesis protocol (Figure 2.9 a). Early phase expression of GATA2 was significantly increased to 30 fold while Wnt3a was increased significantly to 50 fold when estimated quantitatively after 48 hours rohitukine co-incubation (Figure 2.9 d). These all studies indicate that overall adipogenic programming is perturbed in favor of anti-adipogenic activity.
PI3K-AKT pathway is involved in wide variety of metabolic actions. AKT pathway gets up-regulated in cancer and considered major reason for malignancy [63]. Rohitukine analogue flavopiridol exerts anti-cancer activity through inhibiting AKT signaling pathway [64]. AKT/mTOR play an important role in adipogenesis [65]. A representative pathway showing the role of AKT/mTOR pathway in obesity and cell cycle regulation is shown in figure 2.11. Adipogenic stimuli strongly induce AKT phosphorylation which is sustained upto 2 hours. Rohitukine (20µM) co-incubation leads to significant suppression of AKT phosphorylation at Ser-473 residue. It was also found that mTOR phosphorylation at Ser-2448 is also reduced significantly. Rohitukine abrogates phosphorylation of substrate of AKT/mTOR pathway, 4EBP at Thr37/46 which is necessary for protein synthesis during adipogenesis. Rohitukine exerts anti-adipogenic effect in very early phase signaling through PI3K/AKT/mTOR pathway (Fig 2.9 c).

Figure 2.11: Role of AKT/mTOR pathway in obesity and cell cycle regulation

These changes in early signaling upon hormonal induction are of importance which allows fully confluent cells to re-enter into clonal expansion. In view of early blocking in adipogenesis as evidence by microscopic images and ORO absorbance and suppression of MDI signaling (Figure
2.8 d-f), we performed cell cycle regulation study. We found concentration dependent cell number increase in S-phase post 24hr of MDI induction (Figure 2.10 a and b).

Activation and assembly of Cyclin D with CDK 4 and 6, Cyclin E with CDK 2, together with degradation of CDK inhibitors is required for G1/S phase progression and completion of MCE. Many natural compounds have been reported to inhibit adipogenesis by affecting MCE. Rohitukine decreased CDK4 and CDK2 and stabilizes P27 levels (Figure 2.10 c) which is also reflected in cell cycle arrest in S phase in flow cytometry data analysis.

Furthermore decreased expression of C/EBP-β expression further strengthens adipogenesis inhibition phenomenon in early stage. We evaluated the oral bioavailability and found that it is readily bioavailable with more than 25.7% absorbed within 30 min duration and remains in circulation for more than 24 hours.

In conclusion, our studies showed that rohitukine possess both anti-adipogenic and anti-dyslipidemic activity. Taken together all these results, rohitukine inhibited early phase adipogenesis by modulation of MDI induce signaling, MCE arrest by modulation of cell cycle regulatory protein. Although, clinical development of rohitukine for these indication and chronic treatment is dependent on many other factors.

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