Bio-assay Guided Isolation of Corchorusin-D
Cancer is one of the leading causes of death in the world, particularly in developing countries (Kinsey et al., 2008). It affects everyone - the young and old, the rich and poor, men, women and children - and represents a great burden on patients, families and societies.

Among different types of cancer, leukemia causes almost one-third of all cancer deaths in children and adolescents younger than 15 years. On the other hand cancer of the skin is by far the most common of all cancers. Melanoma accounts for less than 5% of skin cancer cases but causes a large majority of skin cancer deaths. (http://www.cancer.org/Cancer/SkinCancer-Melanoma/DetailedGuide/melanoma-skin-cancer-key-statistics). We therefore decided to concentrate on unraveling new therapies for these two types of cancer. Although several modes of treatment are available as discussed in chapter 1, our interest was on the vast pool of natural products considering the advantages they seem to offer.

Nature has been a source of medical treatments for thousands of years and plant based systems continue to play an essential role in health care of 80% countries (Grover and Vats, 2001). Our forefathers recommended some of the substances found abundantly in nature long before their value was demonstrated and understood by scientific methods. India is a country with a vast reserve of natural resources and a rich history of traditional medicines. Indian herbs are used as drugs and remedies to cure and mitigate diseases. Global demand for herbal products has steadily increased during last two decades. Consequently, there has been extensive research on isolation and characterization of compounds from medicinal plants. The alcoholic extract of the whole plant of Corchorus acutangulus was reported to have some anticancer activity therefore the crude methanolic extract of the plant was investigated by us for its potential to inhibit the leukemic cell lines. It demonstrated significant inhibition of cell growth. Based on this observation we proceeded further to isolate and identify the active constituents. Bio-assay guided fractionation and isolation approach was used.
Fig 2.1: Corchorus acutangulus Lam.

Kingdom : Plantae
Subkingdom : Tracheobionta
Superdivision : Spermatophyta
Division : Magnoliophyta
Class : Magnoliopsida
Subclass : Dilleniidae
Order : Malvales
Family : Tiliaceae
Genus : Corchorus
Species : acutangulus

The plants are tall, usually annual herbs, reaching a height of 2-4 m, unbranched or with only a few side branches. The leaves are alternate, simple, lanceolate, 5-15 cm long, with an acuminate tip and a finely serrated or lobed margin. The flowers are small (2-3 cm diameter) and yellow, with five petals; the fruit is a many-seeded capsule. It thrives
General Introduction

Bio-assay Guided Isolation

almost anywhere, and can be grown year-round. The genus *Corchorus* belonging to the family Tiliaceae is distributed throughout the tropical and subtropical regions of the world (Kundu, 1951; Purseglove, 1968; Chang and Miau, 1989). Although 215 species, subspecies, varieties, and forms have been reported under the genus, the precise number of good species is approximately 100 (Saunders, 2006). Out of 100 good species, *Corchorus capsularis* and *Corchorus olitorius* were selected and domesticated in the wake of civilization and are the commercially important cultivated species of *Corchorus*. Wild *Corchorus* taxa are mostly distributed in the tropical/subtropical regions of Africa, America (including Brazil, Mexico, Bolivia, Venezuela, and West Indies), Australia, China, Taiwan, India, Myanmar, Bangladesh, Nepal, Sri Lanka, Japan, Indonesia, Thailand, Malaysia, and Philippines (http://www.gbif.org). In Asia, wild species are distributed in India, Bangladesh, Pakistan, Thailand and Indonesia, but none of them are native to this continent.

*Corchorus acutangulus* Lam. (syn: *C. aestuans*) [Fig 2.1] is a small annual herb belonging to the family Tiliaceae growing throughout the hotter parts of the Indian subcontinent, Indo-China, Australia, Tropical Africa, West Indies and Central America (Ali and Nasir, 1974). In Ayurveda, roots and leaves of *C. acutangulus* are said to cure gonorrhea. The seeds are stomachic and used in the treatment of pneumonia (Ali and Nasir, 1974). The aerial parts contain triterpenoidal glycosides named corchorusins (Ali and Nasir, 1974). Corchorusins have structural similarity with saikosaponins (Mahato and Pal, 1987; Hsu et al., 2000), which have been reported to possess potent anti-tumor activity (Hsu et al., 2004a, 2004b; Sun et al., 2009; Bachran et al., 2008). Saponins are a group of naturally occurring plant glycosides, characterized by their strong foam-forming properties in aqueous solution. The presence of saponins has been reported in more than 100 families of plants out of which at least 150 kinds of natural saponins have been found to possess significant anti-cancer properties. There are more than 11 distinguished classes of saponins including dammaranes, tirucallanes, lupanes, hopanes, oleananes, taraxasteranes, ursanes, cycloartanes, lanostanes, cucurbitanes and steroids (Man et al., 2010). Saikosaponins are a group of structurally modified saponins of oleanane group having ether linkage between C-13 and C-28. Due to the great variability of their
structures, saponins including sikosaponins always display anti-tumorigenic effects through varieties of antitumor pathways.

The alcoholic extract of the whole plant of *C. acutangulus* was reported to have anti-cancer activity against epidermal carcinoma of nasopharynx in tissue culture (Khan *et al.*, 2006). This encouraged us primarily to carry out a thorough chemical analysis of the plant guided by bio-assays. Additionally, alcoholic extract and the glycosides of seeds exhibit cardiotonic activity (Khare, 2007).
MATERIALS AND METHODS

General experimental procedures

Optical rotation was recorded in MeOH using a Jasco P-1020 polarimeter. IR spectra were recorded with a Jasco-FT-IR-model 410 spectrophotometer as KBr disks. $^1$H NMR, $^{13}$C NMR were recorded on a Bruker Ultrashield NMR (600 MHz) in CD$_3$OD with TMS as internal standard. ESI-MS was recorded on a Q-TOF micromass spectrometer. Silica gel (Merck, 100-200 mesh) was used for column chromatography; silica gel (60 F254) was used for TLC and spots were visualised by spraying with Lieberman-Burchard reagent followed by heating. HPLC analyses were performed at 25 ± 1°C using sample solutions filtered through 0.45 µm membrane (Whatman’s syringe filter) and analysed (20 µL injected volume) using a Shimadzu (Japan) system equipped with SCL-10A VP Shimadzu system controller, SPD-M10A VP Shimadzu diode array detector, LC-10AT VP Shimadzu liquid chromatography pump, Class VP software, and a Rheodyne injector with 20 µL loop.

Plant material

Fresh leaves of the plant were collected from around Kolkata, India and authenticated by Botanical Gardens, Howrah, India. A voucher specimen has been deposited at the institute herbarium for future reference.

Extraction and Fractionation

The fresh leaves of the plant were subjected to air drying at room temperature and ground to a coarse powder using a mechanical grinder. The powdered plant material (2 kg) was subjected to extraction using methanol (5 L × 4) for 72 h at room temperature. The extract was then filtered and evaporated to dryness under reduced pressure using rotary evaporator at 45°C to afford the crude methanolic extract (ME, 154 g). ME (113 g) was suspended in water and extracted successively with ethyl acetate and n-butanol, and the
extracts were evaporated to dryness in a rotary evaporator to afford the ethyl acetate (25 g), n-butanol (55 g) and aqueous (30 g) fractions. Each fraction was tested for anti-leukemic activity, and the n-butanol fraction was found to be the most active.

**Isolation and characterisation**

A part (20 g) of the n-butanol fraction was subjected to silica gel column chromatography eluting with increasing polarity of solvents - chloroform followed by chloroform: methanol in the ratios 9:1 (fr-1; 5.5 g), 8:2 (fr-2; 7.3 g), 7:3 (fr-3; 3.5 g), 6:4 (fr-4; 2 g). Each fraction was tested for anti-leukemic activity. Fraction 3 showed the greatest activity among these tested fractions, and was purified by repeated column chromatography over silica gel eluting with chloroform:methanol (4:1) to give a saponin mixture. Preparative HPLC of the mixture (0.45 g) using Waters X Terra™ RP C18 column (19 × 300 mm, 10 μm), using the mobile phase methanol: water (7:3 v/v) at a flow of 20 mL/min and monitoring the eluate at 210 nm to produced three chromatographically pure compounds designated as compound A (30 mg), compound B (40 mg) and compound C (80 mg) [Fig 2.2]. Compound C, which showed significant activity was identified as Corchorusin D (COR-D). The identity of COR-D was confirmed by comparing its physical data as well as its infrared (IR), $^1$H NMR, $^{13}$C NMR and ESI Q-TOF mass spectrometry data with those of an authentic sample available in laboratory and comparing with those in literature (Mahato and Pal, 1987).

**Quantitative analysis by HPLC-DAD**

Separation was achieved using Waters X Terra™ RP C18 column, 4.6× 250 mm, 5 μm particle size; isocratic elution was done using mobile phase methanol:water (7:3 v/v) at a flow rate of 1 mL/min. The eluate was monitored at 210 nm. For HPLC analysis the methanolic extract and fractions were accurately weighed and dissolved in methanol to get a concentration of 10 mg/mL. The isolated constituents were more than 98% pure as determined by HPLC and their standard solutions were prepared in methanol (1 mg/mL). Quantitative estimation of the active constituent/s present in the methanolic extract and
Materials and Methods

Bio-assay Guided Isolation

fractions was done by using the calibration curve of the standard solution (Microsoft Office Excel 2007).

Fig 2.2: Isolation and purification of corchorusin-D (COR-D) from *Corchorus acutangulus*. COR-D was isolated by bioactivity guided fractionation followed by column chromatography. It was purified as single compound by HPLC using Xterra column (C18 reverse phase) with solvent system CH$_3$OH:H$_2$O (7:3 v/v) at a flow 20 ml/min using UV detector at wavelength 210 nm where it eluted at 7.8 minutes. By using IR, $^1$H NMR, $^{13}$C NMR, Mass Spectral data this compound was identified as Corchorusin-D.
RESULTS

Characterisation of COR-D

Colorless needles, m.p. 210-212 °C; [α]D + 40° (c 1.2, MeOH); molecular formula: C_{42}H_{68}O_{13}; Q-TOF ESI-MS m/z 803.57 [M+Na]^+; IR: ν_max cm⁻¹ (KBr) 3404, 2926, 2867, 1723, 1643, 1569, 1384, 1070, 618; ¹H-NMR: (CD₃OD) δ: two anomeric proton signals at 4.68 (1H, d, J = 7.5 Hz) and 4.42 (1H, d, J = 7.5 Hz), double bond proton signals at 5.95 (1H, d, J = 10.2 Hz) and 5.40 (1H, d, J = 10.2 Hz), methyl proton signals at 0.87 (3H), 0.94 (6H), 1.00 (3H), 1.04 (3H) and 1.10 (6H). ¹³C-NMR: see Table 2.1.

Table 2.1: ¹³C NMR chemical shift (δ_c) of COR-D.

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*in pyridine-d₅, 99.6 MHz and §in CD₃OD, 75 MHz.
Table 2.2: Concentration of COR-D determined in ME and NBF and purity of isolated sample.

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<th>Sample</th>
<th>Concentration of COR-D (% w/w)*</th>
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<td>Methanolic Extract (ME)</td>
<td>1.38 ± 0.09</td>
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<tr>
<td>n-Butanol Fraction (NBF)</td>
<td>4.22 ± 0.18</td>
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<tr>
<td>COR-D</td>
<td>98.72 ± 0.34</td>
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* Mean ± Standard deviation; n=3.
Fig 2.3: ESI-Mass spectrum of COR-D
Fig 2.4: IR spectrum of COR-D
Fig 2.6: $^{13}$C NMR spectrum of COR-D
Fig 2.7: $^{13}$C NMR spectrum: DEPT-135 of COR-D
Fig 2.9: HPLC chromatogram for ME (A), NBF (B) and isolated COR-D (C). Eluent: Methanol: water (7:3 v/v); flow-rate: 1 ml/min; UV detection: 210 nm. Arrow indicates peak for COR-D.

Fig 2.10: Calibration curve of standard COR-D obtained using HPLC
REFERENCES


Global Biodiversity Information Facility 2008: http://www.gbif.org


Sun Y, Cai TT, Zhou XB, Xu Q (2009) Saikosaponin a inhibits the proliferation and activation of T cells through cell cycle arrest and induction of apoptosis. Int Immunopharmacol 9:978-983