Bioactivity-guided isolation of cytotoxic constituents from three medicinal plants

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Abstract

Context: The ethanol extracts and their fractions of three Indian medicinal plants, *Ervatamia coronaria* (Jacq.) Stapf, (Apoecynaceae), *Mimosa pudica* L. (Mimosaceae) and *Caesalpinia bonduc* (L.) Roxb. (Caesalpiniaceae) were tested for their cytotoxic activity in the brine shrimp lethality (BSL) bioassay and in various cancer cell lines. The plants were selected based on their traditional use in the treatment of cancer/tumors.

Objectives: To investigate the in vitro cytotoxicity of *Ervatamia coronaria*, *Mimosa pudica* and *Caesalpinia bonduc*.

Materials and methods: Ethanolic extracts and their fractions of *E. coronaria*, *M. pudica* and *C. bonduc* were subjected to cytotoxicity studies using BSL bioassay method with concentrations of 10, 50, 100, 500 and 1000 \(\mu\)g/ml. The alkaloid fraction of *E. coronaria* with significant cytotoxicity in BSL bioassay was subjected to in vitro cytotoxicity studies with HT-29, A-549, HepG-2, MCF-7 and L-6 cell lines at concentrations of 12.5, 25, 50, 100 and 200 \(\mu\)g/ml and a DNA fragmentation study using the HT-29 cell line.

Results: The alkaloid fractions of *E. coronaria* and *M. pudica* showed significant cytotoxicity with \(\text{LC}_{50}\) values of 65.83 and 85.10 \(\mu\)g/ml in the BSL bioassay, respectively. The purified alkaloid fraction of *E. coronaria* exhibited highest cytotoxicity in HT-29, A-549 and MCF-7 cell lines with \(\text{IC}_{50}\) values of 32.5, 47.5 and 72.5 \(\mu\)g/ml, respectively, and induced DNA fragmentation in the HT-29 cell line at a concentration of 65 \(\mu\)g/ml.

Conclusion: The alkaloid fraction of *E. coronaria* exhibited significant cytotoxicity. Alkaloids such as ervatamine, apparicine and coronaridine that were earlier reported may be responsible for this activity.

Introduction

Cancer is a group of diseases where a group of cells display uncontrolled growth, invasion and metastasis. According to WHO, cancer is a leading cause of death worldwide. In 2007, around 13% of all deaths were due to cancer (Jemal et al., 2011). It has been estimated that by 2030 deaths due to cancer may be around 12 million. In India, the International Agency for Research on Cancer estimated indirectly that more than half a million people died from cancer in 2008, representing about 8% of all estimated global cancer deaths and about 6% of all deaths in India (Ferlay et al., 2008). The number of cancer deaths in India is projected to increase because of population growth and increasing life expectancy (Jha, 2009).

The estimated cancer mortality in 2000 was over 1.5 million, which is 3.61% of all deaths.

Anticancer agents from plant sources have various modes of action that inhibit various stages of the cancer cell growth. These phytoconstituents may act as anticancer agents either by inhibiting the cell growth or by killing the cancer cells (cytotoxic). The United States National Cancer Institute (NCI) played a major role in screening a large number of anticancer compounds from natural source. In 1955, the NCI set up the Cancer Chemotherapy National Service Center to act as a public screening center for anti-cancer activity of compounds (Goodman & Walsh, 2001). Many of these phytoconstituents have proved to be highly potent anticancer agents such as *Vinca* alkaloids, irinotecan, camptothecins, etoposides and paclitaxel (taxol). The NCI collected about 35,000 plants from 20 countries and has screened around 114,000 extracts for their anticancer activity (Shoeb, 2006). Of the 92 anticancer drugs commercially available prior to 1983 in the US and among worldwide-approved anticancer drugs between 1983 and 1994, 60% are of natural origin (Cragg et al., 1997).

In this context, the present study investigated the aerial parts of *Ervatamia coronaria* (syn. *Tabernaemontana*...
**E. coronaria** Jacq.) Willd. (Apocynaceae), whole plant of *Mimosa pudica* Linn. (Mimosaceae) and root bark of *Caesalpinia bonduc* (L.) Roxb. Dandy & Exell. (syn. *Caesalpinia bonducella* Flem.) (Caesalpiniaceae) for their cytotoxic activity. The extraction and fractionation method was a slight modification of the method proposed by Cos et al. (2006). The leaves and aerial parts of *E. coronaria* have been reported to have anticancer property (Khare, 2007). The whole plant of *M. pudica* is used in the treatment of uterine tumors and root bark of *C. bonduc* is used in the treatment of various tumors in the traditional systems of medicine in India (Kirtikar & Basu, 1999).

Hence, the ethanol extract and their various fractions of these three plants were subjected for cytotoxicity evaluation using the brine shrimp lethality (BSL) bioassay and the fractions with promising results were subjected for *in vitro* cell line studies using MTT assay followed by DNA fragmentation studies.

**Materials and methods**

**Plant material**

The aerial parts of *E. coronaria* were collected from Belgaum surroundings in the month of June 2010; whole plant of *M. pudica* was collected from the vicinity of ICMR Belgaum in the month of June 2010; root bark of *C. bonduc* was collected from Jath, Sangli, Maharashtra in the month of August 2010. Taxonomic identification and authentication was done by Dr. Harsha Hegde (Research Scientist), Indian Council of Medical Research, Belgaum, Karnataka. The voucher specimens (RMRC-546; RMRC-547 and RMRC-556, respectively) are deposited at the Herbarium and crude drug museum of Indian Council of Medical Research, Belgaum, Karnataka along with a sample of crude drug. All the plant materials were washed under running tap water and shade-dried. The shade-dried plant materials were powdered using a dry grinder to obtain coarse powder. The powdered crude drugs were stored in airtight containers separately until further use.

**Cell lines and culture media**

HT-29 (Human, colon cancer), A-549 (Human, small cell lung carcinoma), HepG-2 (Human, hepatic cancer), MCF-7 (Human, breast cancer) and L-6 (Rat, normal skeletal muscle) cell cultures were procured from National Centre for Cell Sciences, Pune, India. Stock cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% inactivated fetal bovine serum (FBS), penicillin (100 IU/ml), streptomycin (100 μg/ml) and amphotericin B (5 μg/ml) in an humidified atmosphere of 5% CO<sub>2</sub> at 37°C until confluent. The cells were dissociated with trypsin phosphate versene glucose solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm<sup>2</sup> culture flasks (Tarsons India Pvt. Ltd, Kolkata, India).

**Extraction and fractionation**

The air-dried powdered plant materials were subjected to maceration using ethanol for 48 h. The extract was filtered and the marc was subjected to Soxhlet extraction using ethanol as a solvent for 10 h. The extract was filtered at elevated temperature and mixed with the extract obtained by maceration. The combined extract was concentrated under reduced pressure at 40°C using a rotary evaporator. The solvent free extract was subjected to fractionation using the protocol given by Cos et al. (2006) with slight modifications. The detailed method is given in the form of a flow chart (Figure 1). This procedure was employed to avoid any possible destruction of chemical constituents due to higher temperature during repeated extraction.

**BSL bioassay**

The brine shrimp (*Artemia salina* Lich.) eggs were obtained from Department of Pharmacology, Manipal College of Pharmacy, Manipal, India. The assay was a test for determination of the cytotoxicity of the extracts/fractions. The procedure and method was as described by McLaughlin et al. (1998). The chamber was divided into two equal parts. Aeration was given in both the compartments. One part was illuminated with a bulb (60 W), while the other was darkened. Surface sterilized brine shrimp eggs were sprinkled in the dark side and incubated at room temperature for 48 h. As the hatching occurred, the nauplii were swum towards the illuminated side, where they were collected by a Pasteur pipette. Samples of the extracts/fractions were prepared by dissolving 50 mg of extract in 5 ml of DMSO to get 5000 μg/ml stock solution and further diluted with seawater to get the required concentrations (10, 50, 100, 500 and 1000 μg/ml). Dried vials were taken and 10 shrimps were transferred in each vial and then the volume was made up to 5 ml with seawater. A drop of dry yeast suspension (3 mg in 5 ml seawater) was added to each vial as food for shrimps. For each concentration tests were carried out in triplicate. Control vials were prepared by adding equal volumes of distilled water. The vials were maintained under illumination. After 24 h survivors were counted, by using 3× magnifying glass and the percentage of mortality and LC<sub>50</sub> values were calculated by Probit analysis using SPSS-10.0.5 software (Armonk, NY).

**MTT assay**

For cytotoxicity studies, a weighed quantity of sample was dissolved in DMSO and the volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two-fold dilutions were prepared from this for carrying out cytotoxic studies.

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 × 10<sup>5</sup>cells/ml using DMEM medium containing 10% FBS. To each well of the 96-well microtiter plate, 0.1 ml of the diluted cell suspension (approximately 10000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, the monolayer was washed once with medium and 100 μl of different test concentrations of fraction was added on to the partial monolayer in microtiter plates. The plates were incubated at 37°C for 3 d in 5% CO<sub>2</sub> atmosphere, and microscopical examination was carried out and observations were recorded at 24 h intervals. After 72 h, drug solutions in the wells were discarded and 50 μl of MTT in PBS were...
added to each well. The plates were gently shaken and incubated for 3 h at 37°C in a 5% CO₂ atmosphere. The supernatant was removed and 100 µl of propanol were added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (IC₅₀) values is generated from the dose-response curves for each cell line (Danizot & Lang, 1986).

\[ \text{Growth inhibition} = 100 - \left( \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \right) \times 100 \]

**DNA fragmentation studies**

HT-29 cells (3 × 10⁶/ml) were seeded into 60 mm plates and incubated at 37°C with a 5% CO₂ atmosphere for 24 h. The cells were washed with medium and treated with double the IC₅₀ value of fraction (65 µg/ml), standard drug (30 ng/ml) and incubated at 37°C, 5% CO₂ for 24 h. As the incubation time ended, the chromosomal DNA of cancer cells was prepared with Roche apoptotic DNA ladder kit. Briefly, cells were harvested and lysed with lysis buffer for 10 min. The samples were mixed with isopropanol before passing through the filter and washed. The DNA was eluted from the filter and treated with RNAs at 37°C for 30 min before loading onto 2% agarose gel electrophoresis and run 50 V/cm for 3 h. The gel was visualized under an UV trans-illuminator and photographed (Wan et al., 2005).

**Data analysis**

The LC₅₀ values were calculated by Probit analysis at 95% confidence level using SPSS-10.0.5 statistical software.

**Results**

**Phytochemical screening**

All the extracts and the fractions were subjected to preliminary phytochemical investigations. Fractions I and II of all the extracts were found to contain highly non-polar constituents such as steroids and triterpenoids. Fraction III of both *M. pudica* and *E. coronaria* extract showed the presence of alkaloids. Fraction IV of all the extracts contained polyphenols and flavonoids. Saponins were found in fraction IV of *E. coronaria* (Table 1).

**BSL bioassay**

All the three extracts and their fractions were subjected to the BSL bioassay at concentrations of 10, 50, 100, 500 and 1000 µg/ml. The alkaloidal fractions (Fraction III) of *E. coronaria* and *M. pudica* exhibited cytotoxicity with a LC₅₀ values of 65.83 and 85.10 µg/ml, whereas other extracts and fractions showed moderate to no cytotoxicity in the BSL.
The % yield of the extract was calculated based on the weight of air-dried plant material and % yield of the fractions was calculated based on dry weight of the respective extracts.

Table 2. Effect of different extracts/fractions with the BSL bioassay.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Extract/fraction</th>
<th>Mean % death after 24 h (concentration in μg/ml)</th>
<th>LC50 (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>Control</td>
<td>–</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td><em>E. coronaria</em> (aerial part)</td>
<td>Eth. Ext</td>
<td>0.00</td>
<td>10.52 ± 0.98</td>
</tr>
<tr>
<td></td>
<td>Frac. 1</td>
<td>0.00</td>
<td>9.56 ± 0.65</td>
</tr>
<tr>
<td></td>
<td>Frac. 2</td>
<td>23.36 ± 1.65</td>
<td>31.54 ± 1.58</td>
</tr>
<tr>
<td></td>
<td>Frac. 3</td>
<td>19.65 ± 1.23</td>
<td>47.54 ± 2.68</td>
</tr>
<tr>
<td></td>
<td>Frac. 4</td>
<td>0.00</td>
<td>9.31 ± 0.89</td>
</tr>
<tr>
<td><em>M. pudica</em> (whole plant)</td>
<td>Eth. Ext</td>
<td>9.87 ± 0.75</td>
<td>11.21 ± 1.02</td>
</tr>
<tr>
<td></td>
<td>Frac. 1</td>
<td>0.00</td>
<td>9.92 ± 0.88</td>
</tr>
<tr>
<td></td>
<td>Frac. 2</td>
<td>5.60 ± 0.45</td>
<td>11.21 ± 0.96</td>
</tr>
<tr>
<td></td>
<td>Frac. 3</td>
<td>7.32 ± 0.32</td>
<td>25.63 ± 1.96</td>
</tr>
<tr>
<td></td>
<td>Frac. 4</td>
<td>8.60 ± 0.65</td>
<td>10.32 ± 1.02</td>
</tr>
<tr>
<td><em>C. bonduc</em> (root bark)</td>
<td>Eth. Ext</td>
<td>9.92 ± 0.72</td>
<td>15.02 ± 1.21</td>
</tr>
<tr>
<td></td>
<td>Frac. 1</td>
<td>0.00</td>
<td>9.32 ± 0.65</td>
</tr>
<tr>
<td></td>
<td>Frac. 2</td>
<td>6.5 ± 0.52</td>
<td>11.02 ± 0.85</td>
</tr>
<tr>
<td></td>
<td>Frac. 3</td>
<td>9.98 ± 0.32</td>
<td>11.03 ± 0.75</td>
</tr>
<tr>
<td></td>
<td>Frac. 4</td>
<td>0.00</td>
<td>10.05 ± 0.79</td>
</tr>
</tbody>
</table>

All the values are mean ± SEM of three samples.

bioassay (Table 2). With promising results from the BSL assay, the *E. coronaria* alkaloidal fraction (Fraction III) was further purified and tested for cytotoxic activity in different in vitro models.

**MTT assay**

Since the alkaloidal fraction of *E. coronaria* extract revealed potent cytotoxicity in the BSL bioassay it was further purified and subjected for in vitro cytotoxicity studies with various cancer cell lines using the MTT assay (Figure 2). The percentage cell death was tested for each cell line after 72 h of incubation. The fraction inhibited the proliferation of cells in a dose-dependent manner in the concentration range of 12.5–200 μg/ml with significant cytotoxicity at all the five tested cell lines. The activity was more prominent in HT-29 (human, colon cancer) and A-549 (human, small cell lung carcinoma) cell lines with IC50 (concentration required to inhibit 50% of cell growth) values of 32.5 and 47.5 μg/ml, respectively. In other cell lines it showed moderate cytotoxicity (Table 3).
was selected for further confirmation of cytotoxic activity using five different cancerous cell lines. The total alkaloidal fraction of *E. coronaria* showed significant cytotoxicity in human colon cancer (HT-29). Human small cell lung carcinoma (A-549) and human breast cancer cell lines (MCF-7) were also found sensitive to *E. coronaria* alkaloids. Two other cell lines, human hepatic cancer (HepG-2) and rat normal skeletal muscle (L-6), were comparatively less sensitive to these alkaloids (Figure 3). The different parts of *E. coronaria* are used traditionally for the treatment of cancer (Khare, 2007). The results of our study provides scientific validation for the traditional claim.

Based on the cell line study data, we further proceeded with DNA fragmentation studies using HT-29 cell lines. The study was conducted simultaneously with paclitaxol which is a clinically proven anticancer agent. The results were again encouraging showing the clear fragmentation of the HT-29 DNA.

Various authors have reported a number of alkaloids from *E. coronaria*. A bisindole alkaloid, 19, 20-dihydroervahamine A was isolated from the stems of *E. coronaria* grown in Brazil (Henriques et al., 1996). Knox and Slobbe (1971) isolated ervatamine an β-acyl-indolic alkaloid for the first time. Another indole alkaloid, apparicine, was isolated from the leaves of *E. coronaria* (Atta-ur-Rahaman et al., 1984). Coronaridine, an ibogamine related alkaloid, has been isolated from *Ervatamia* sp. (Gormam et al., 1960). A major indole alkaloid, vovacristine, has been isolated from *E. coronaria* and it has exhibited a cytostatic, cytotoxic and mutagenic effect in wild-type and repair-deficient yeasts (Melo et al., 1986).

Most of the clinically proved cytotoxic compounds are indole alkaloids, including *Vinca* alkaloids (Feng et al., 2010). The ibogan type, coronaridine, showed appreciable cytotoxicity toward sensitive (KB/S) as well as vincristine-resistant (KB/VJ300) cells (Kam et al., 2004). Six vobasinyl ibogan type bisindole alkaloids including four new ones, ervachinines A-D, were isolated from whole plant together with 10 known monoterpoid indole alkaloids and found to be quite potent in terms of cytotoxic activity (Guo et al., 2012). These data indicate that the alkaloids of *E. coronaria* may contain a useful cytotoxic component that needs further study regarding anticancer properties.

### Conclusions

The results of studied parameters confirm the potential of these three taxa for the production of cytotoxic components.
This report provides very important data regarding the alkaloids of *E. coronaria* as a potent cytotoxic component. Further isolation, identification and antineoplastic studies of these compounds comprise ongoing investigations.

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**Declaration of interest**
The authors report no conflicts of interest.

**References**


