Investigation of phytochemical constituents of anti-leukemic herbal drugs used by the traditional healers of Purulia, Birbhum and Bankura districts of West Bengal

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Abstract

In the present work sixteen different plant drugs used by traditional healers from West Bengal were screened through in vitro cell line model. Herbal drugs used by traditional tribal healers in Purulia, Birbhum and Bankura districts of West Bengal were collected and screening against acute myeloid leukemia (AML) cell line (HL-60). Among sixteen plant extracts, bark of Flacourtia indica (66.67%), leaf of Madhuca longifolia (69.17%), and leaf of Prosopis cineraria (68.08%) showed better cytotoxicity results than other herbals. Further, time-dependent study showed maximum cytotoxicity of the selected herbal extracts between 36 - 48 hours of treatment in both acute and chronic myeloid leukemia (CML) cell lines (HL-60 and K562). The LC-MS/MS analysis of the selected drugs revealed the presence of picrotoxinin and 10-deacetylbaccatin from F. indica, isoorientin and hirsutrin from M. longifolia, vitexin and rhoifolin in P. cineraria.
Experimental

Collection of samples

Information about the herbal drugs, dosage level and mode of administration were obtained from the local traditional healers in the Purulia, Birbhum and Bankura districts of West Bengal with the help of Dr. Sukanta Hazra, Ayurvedic Doctor, Government Hospital, Shibpur, Howrah. All the herbals collected from traditional healers were identified and authenticated by Botanical Survey of India (SBI), Howrah, West Bengal and Centre for Advanced Research in Indian System of Medicine (CARISM), SASTRA Deemed University, Thanjavur. All the materials were shade dried, powdered and used for preparing the extract.

Preparation of decoction

Decoction of selected drug materials was prepared by taking 25 g of herbal samples with 250 ml of distilled water in flasks and boiled on a hot plate until the volume reduced to ¼ of the original volume. Then the content was filtered and the filtrate was lyophilized and re-dissolved in distilled water at 5 mg/ml concentration and membrane filtered through Nupore (0.45 µ) and used for cell culture experiments.

Cytotoxicity

Leukemia cell lines HL-60 and K-562 were obtained from National Centre for Cell Sciences (NCCS), Pune, India was used for the basic screening of cytotoxicity of herbal extracts using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) cell viability assay. Briefly 10^4 cells were taken per well in a 96 well plate, cultured in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin (Himedia A028), and maintained in a 5% CO₂ incubator (NBS-Eppendorf, Galaxy 170S) at 37°C. The cells were treated with different concentrations of herbal extracts (125, 250 and 500 µg/ml) and incubated for 48 h at 37°C. After that, 20 µl MTT solutions (5 mg/ml) was added per well and incubated at 37°C for 4 h followed by solubilizing the formazan product in an 4 mM HCl-isopropanol solution. Then the absorbance was measured using a micro plate reader (Tecan Sunrise ELISA reader) at 595 nm. Based on the data the cytotoxicity was calculated and expressed on percentage basis.

Time-dependent cytotoxicity

After the basic screening, aqueous extract of Flacourtia indica bark, Madhuca longifolia leaves, Prosopis cineraria leaves were found to exhibit high level of cytotoxicity. In order to check their time-dependent cytotoxicity, we have investigated the cytotoxicity against both AML (HL-60) and CML (K-562) cell lines using MTT assay at different time points (12, 24, 36 & 48 h).
Safety studies against PBMC

In vitro toxic effect of herbal extracts was tested against human peripheral blood mononuclear cells (PBMC). PBMC were isolated from the healthy volunteers using Histopaque (Sigma, 10771) and re-suspended in RPMI-1640 media (supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 unit/ml penicillin and 100 µg/ml streptomycin) and maintained in a 96-well plate at a density of $10^5$ cells/well. PBMCs were treated with different concentrations of herbal extracts (100, 200, 400, 800 and 1600 µg/ml) and incubated for 24 h at 37°C in CO$_2$ incubator. Then, 20 µl MTT solution (5 mg/ml) was added per well and incubated at 37°C for 4 h and the formazan product was solubilized in 4 mM HCl-isopropanol solution. The absorbance was measured using a micro plate reader (ELISA reader, Tecan Sunrise) at 595 nm and the cell viability was calculated.

LC-MS analysis

Aqueous extracts of *F. indica*, *M. longifolia* and *P. cineraria* were filtered using 0.45 µm syringe filter and analyzed using liquid chromatography coupled to mass spectrometer (LC/ESI/MS/MS, MicroTOF-Q II, Bruker, Germany) to identify the phytochemical constituents. Solution (10 µl) was injected for liquid chromatography separations in a C18 reverse phase column (120 Å, 2.1 x 150 mm, 3.0 µm, Dionex, USA). UV detector was set arbitrarily at 330 nm. A discontinuous gradient elution at a flow rate of 0.2 ml/min was performed using mobile phase A represent acetonitrile and mobile phase B represent water (MilliQ) acidified with acetic acid (1%). The gradient started from 1% of A for 0.2 min and it was then brought to 75% A at 16th min and then reaching at 100% A at 19th min to 5% A at 21st min and was maintained at same condition till run ends at 30th min. Eluted compounds were then identified using MS and their respective MS/MS pattern. Optimized parameters consisted in collision energy 10 eV, focusing potential of 350 voltage per peak, transfer time of 800 µs, pre-pulse storage of 5 µs the instrument was operated in the negative ion mode with a capillary voltage of 4.5 KV, capillary temperature was 270°C, sheath gas (N2) flow rate was 6 ml/min with 30.5 psi pressure. The results of molecular mass were compared with mass bank data and the phytochemicals were identified.

In silico approach

Based on the major compounds identified from the aqueous extract of *F. indica*, *M. longifolia*, and *P. cineraria* molecular docking studies were carried out with AutoDock 4.0 software to analyze ligand interactions with the crystal structure binding site of BCR-ABL (PDB ID: 2ABL) and KRAS (PDB ID: 5UK9) obtained from protein data bank (PDB). The 3-D grid box has been generated with a grid centre co-ordinates comprising of grid spacing
0.375 Å and $70 \times 70 \times 70$ point size considering active site residues included within it. For preparation of the AutoDock docking parameter file, we have used default settings (Genetic algorithm parameters: population size 150, number of energy evaluations $2.5 \times 10^7$, rate of gene mutation 0.02, rate of crossover 0.8, maximum number of generations 27000, number of GA runs 10) and initial dihedrals were randomly specified, elitism value was set to 1 (Dandwate et al. 2012). Results were visualized using Ligplot+ (EMBL-EBI).
### Supplementary Table S1. List of anticancer drugs used by traditional healers of tribal communities in West Bengal

| S. No. | Botanical name                  | Family       | Bengali name | Plant part used | Dosage level       |
|--------|---------------------------------|--------------|--------------|-----------------|--------------------|
| 1      | *Acorus calamus* L.             | Acoraceae    | *Vachaa*     | Dried rhizome   | Decoction, 25-50 ml/day |
| 2      | *Alpinia zerumpet* (Pers.) B. L. Burtt (Voucher No. BSI/CDM/021a) | Zingiberaceae | *Champa*     | Dried rhizome   | Decoction, 20 ml/day   |
| 3      | *Allium sativum* L.             | Amaryllidaceae | *Lahsuna*   | Dried bulb      | Decoction, 50 ml/day   |
| 4      | *Andrographis paniculata* Burm. f. (Voucher No. CARISM/00119) | Acanthaceae  | *Kalmegh*    | Leaves          | Decoction, 10-20 ml/day |
| 5      | *Berberis vulgaris* L.          | Berberidaceae | *Darhaldi*   | Bark            | Decoction, 20 ml/day   |
| 6      | *Boswellia serrata* Triana & Planch. (Voucher No. CARISM/00124) | Burseraceae  | *Bhishal*    | Resin           | Decoction, 30-50 ml/day |
| 7      | *Commiphora mukul* Hook.        | Burseraceae  | *Guggul*     | Gum resin       | Decoction, 50 ml/day   |
| 8      | *Embelia ribes* Burm. f.        | Primulaceae  | *Biranga*    | Seeds           | Decoction, 15-25 ml/day |
| 9      | *Flacourtia indica* Burm. f. Merr. (Voucher No. CARISM/00121) | Salicaceae   | *Baichi*     | Bark            | Decoction, 10 ml/day    |
| 10     | *Foeniculum vulgare* Mill.      | Apiaceae     | *Saunf*      | Seeds           | Decoction, 10-20 ml/day |
|   | Species                          | Family            | Common Name | Part Used | Preparation | Dosage          |
|---|----------------------------------|-------------------|-------------|-----------|-------------|----------------|
| 11| *Myristica fragrans* Houtt.      | Myristicaceae     | Jaatipatra  | seeds, aril | Decoction   | 50 ml/day      |
|   | (Voucher No. BSI/CDM/232)        |                   |             |           |             |                |
| 12| *Madhuca longifolia* J. Konig   | Sapotaceae        | Mahua       | Leaves    | Decoction   | 20 ml/day      |
|   | (Voucher No. BSI/CDM/273)        |                   |             |           |             |                |
| 13| *Piper longum* L.               | Piperaceae        | Pippali     | Fruits    | Decoction   | 25-50 ml/day   |
|   | (Voucher No. BSI/CDM/267)        |                   |             |           |             |                |
| 14| *Prosopis cineraria* (L.) Druce | Fabaceae          | Shami       | Leaves    | Decoction   | 50 ml/day      |
|   | (Voucher No. BSI/CDM/378)        |                   |             |           |             |                |
| 15| *Solanum nigrum* L.             | Solanaceae        | Kakmachi    | Dried fruits | Decoction   | 10-20 ml/day   |
|   | (Voucher No. CARISM/00122)       |                   |             |           |             |                |
| 16| *Solanum torvum* Sw.            | Solanaceae        | Tit Begun   | Dried fruits | Decoction   | 10 ml/day      |
|   | (Voucher No. BSI/CDM/323)        |                   |             |           |             |                |
Supplementary Table S2. Autodocking results of major compounds identified from the aqueous extract of *Flacourtia indica*, *Madhuca longifolia* and *Prosopis cineraria* against leukemia targets.

| Plant Name / Drug       | Compound   | Target      | Binding energy | Intermolecular energy |
|-------------------------|------------|-------------|----------------|-----------------------|
| *Flacourtia indica*     | Picrotoxinin | K-RAS      | -5.47          | -6.84                 |
|                         |            | BCR-ABL     | -5.17          | -6.54                 |
|                         | 10-Deacetylbaccatin | K-RAS | -           | -                      |
|                         |            | BCR-ABL     | -             | -                      |
| *Madhuca longifolia*    | Isoorientin | K-RAS      | -2.66          | -3.82                 |
|                         |            | BCR-ABL     | -5.52          | -6.90                 |
|                         | Hirsutrin  | K-RAS      | -5.93          | -8.90                 |
|                         |            | BCR-ABL     | -5.73          | -8.62                 |
| *Prosopis cineraria*    | Vitexin    | K-RAS      | -5.06          | -7.71                 |
|                         |            | BCR-ABL     | -6.20          | -8.97                 |
|                         | Rhoifolin  | K-RAS      | -             | -                      |
|                         |            | BCR-ABL     | -             | -                      |
| Clinical drug           | Doxorubicin| K-RAS      | -6.04          | -9.42                 |
|                         |            | BCR-ABL     | -5.85          | -6.04                 |
Supplementary Figure S1. Screening results on the cytotoxicity of aqueous extract of phyto-drugs.
Supplementary Figure S2. Time-dependent cytotoxicity of aqueous extracts of *Flacourtia indica*, *Madhuca longifolia* and *Prosopis cineraria* against acute leukemia cells HL-60 (A) and chronic leukemia cells K562 (B).
Supplementary Figure S3. Cytotoxicity of herbal extracts against peripheral blood mononuclear cells (PBMC).
Supplementary Figure S4. LC-MS profile of aqueous extract of *Flacourtia indica* bark.

**LC-MS profile of Flacourtia indica**

![LC-MS profile of Flacourtia indica](image)

**Picrotoxinin**

Cmpd 5, -MSn(291.2), 36.1 min

![Picrotoxinin](image)

**10-Deacetylbacatin**

Cmpd 3, -MSn(543.1), 4.8 min

![10-Deacetylbacatin](image)
Supplementary Figure S5. LC-MS profile of aqueous extract of *Madhuca longifolia* leaves.

**LC-MS profile of Madhuca longifolia**

**Iso-orientin**

Compd 62, -MSn(447.1), 5.4 min

**Hirsutrin**

Compd 65, -MSn(463.1), 5.5 min
Supplementary Figure S6. LC-MS profile of aqueous extract of *Prosopis cineraria* leaves.

**LC-MS profile of *Prosopis cineraria***

**Vitexin**

*Compd 24, -MSn(431.1), 2.8 min*

**Rhoifolin**

*Compd 29, -MSn(577.2), 3.2 min*
Supplementary Figure S7. Structure of major phytochemicals identified in selected herbal extracts through LC-MS/MS analysis.

*F. indica*
- 10-Deacetylbaccatin
- Picrotoxinin

*M. longifolia*
- Isoorientin
- Hirsutin

*P. cineraria*
- Vitexin
- Rhoifolin
Supplementary Figure S8. Binding affinity of ABL-BCR and KRAS proteins with phytochemicals.