Effect of methanolic and water extract of *Leucobryum bowringii* Mitt. on growth, migration and invasion of MCF 7 human breast cancer cells *in vitro*

G S Manoj¹, T R Santhosh Kumar², Saneesh Varghese³ & K Murugan⁴ *

¹,⁴ Plant Biochemistry and Molecular Biology Laboratory, Department of Botany, University College, Thiruvananthapuram 695 034, India
²,³ Rajiv Gandhi Center for Biotechnology, Poojapura, Thiruvananthapuram 695 014, India

*Received 17 January 2012; revised 22 June 2012*

Inhibitory effects of methanol and water extract of *L. bowringii*. on the adhesion, migration, invasion and matrix metalloproteinase (MMP) activities of MCF 7 human breast cancer cell line are reported. Cells were cultured with 10, 25, 50 µg/mL methanolic or water extract of *L. bowringii*. Culture medium containing 0.1% DMSO was used as a solvent control. Ultra structural analysis by electron microscopy revealed typical features of apoptosis. A remarkable dose-response parallelism was observed between methanolic extract with growth, migration and invasion of breast cancer cells. Fractionation of methanolic extract by RP-HPLC revealed a pool of phenolic acids. Hoechst 33342 staining assay reveals massive chromatin condensation and subsequent cleavage of structural components of nucleus. The results indicate that methanol extracts inhibit the growth of human breast cancer cells partially through the inhibition of metallo proteinases MMP-2 and MMP-9 activities. Methanolic extract has more anti-metastatic effects in cell based assay than water extract. Clinical application of *L. bowringii* extract as a bioactive chemopreventive compound may be helpful in limiting breast carcinoma invasion and metastasis.

**Keywords:** Adhesion, Breast cancer cells, Invasion, *Leucobryum bowringii*, Metalloproteinase, Migration

Breast carcinoma represents the major cause of death among women worldwide due to its high metastatic capability¹. Metastasis is a series of events including the detachment of tumor cells from the primary site, adhesion, migration and invasion of tumor cells into the blood or lymphatic vessel, extravasations out of the vessel, and interactions with the target tissues. Motility and invasion into the new target tissue result in the formation of a secondary tumor². These stages are determined by characteristics inherent to the tumor cells, such as hormone receptor status, production of proteolytic enzymes, expression of cell adhesion molecules and increase in motility³ as well as by a number of growth factors, matrix molecules and cytokines in the metastatic microenvironment⁴.

Matrix metalloproteinases (MMPs) are related to tumor invasion and metastasis because of their capacities for tissue remodeling *via* the extracellular matrix, basement membrane degradation and induction of angiogenesis⁵. In this respect, a correlation between a high expression of MMP-2 and reduced survival in breast cancer patients, as well as an association of the tumor grade with increased levels of MMP-9 in breast cancer tissue was described. Efficient reduction of MMP-2 and 9 levels was observed during *in vitro* treatment of MCF 7 breast cancer cells with the aromatase-inhibitor letrozole, suggesting that this inhibitor suppresses both breast cancer growth and invasion⁶. Conventional therapeutic strategies include surgery, radiation, and chemotherapy. The chemotherapeutic drugs that have been observed to induce apoptosis *in vitro* include etoposide, camptothecin, VM26, vincristine, cisplatin, cyclophosphamide, paclitaxel, 5-fluorouracil and doxorubicin⁷. Due to their side effects and limited effectiveness, there is still a pressing need for the development of anti-breast cancer drugs. Natural products exerting diverse bioactivities and possessing unique structural properties are an important source for the development of novel anticancer drugs.

Bryophytes are traditional Japanese medicinal herbs extensively used to treat skin allergies, to protect the liver and to treat hepatitis beside, being used as antipyretics. These biological activities of bryophytes are triggered from the active ingredients

*Correspondent author
Telephone: 9447077895
E-mail: harimurukan@gmail.com*
present in the cells such as sesquiterpenes, diterpenes and lipophilic aromatics\(^8\). \textit{Leucobryum bowringii}, is a whitish moss that grows in dense cushion with long spongy leaves, in dampy soil or as epiphytes. Cordell \textit{et al.}\(^9\) reported anti-tumor promoting activity of \textit{Leucobryum bowringii} Mitt. a bryophyte by Muthuvan Tribes, Kerala. No scientific validation is available related to the therapeutic potentiality of this plant. Therefore, anti-metastatic effects of methanolic and water extracts of \textit{L. bowringii} have been studied on the adhesion, migration, invasion and MMP activity of MCF 7 human breast cancer cells.

Materials and Methods

Preparation of the extract—Fresh leafy shoots of \textit{Leucobryum bowringii} Mitt. were collected from the Kellar river floor of Ponmudi Hills, Thiruvananthapuram, Kerala, India. Taxonomic identity was confirmed by comparing with authenticated herbarium specimen at Department of Botany Herbaria, University of Calicut, Kerala. A voucher specimen of the plant was kept in the herbarium of the institute (UC DB 234).

Fresh leafy shoots were (100 g) chopped and successively extracted with 300 mL of methanol and water for 6 h using soxhlet hot extraction method. The supernatants were concentrated using rotavapour at 50 °C. The yields of the methanolic and water extracts were 7.4 g and 6.2 g respectively. The methanolic and water residues were lyophilized and stored at -20 °C. Different concentrations of lyophilized methanolic or water extracts were prepared by dissolving in 0.1 % dimethyl sulfoxide (DMSO).

Cells and culture conditions—The MCF 7 human breast cancer cells were obtained from the American Type Culture and Collection (Rockville, MD). All cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Gaithersburg), supplemented with penicillin (100 units/mL), streptomycin 100 (µg/mL) and 10% fetal bovine serum, and incubated in a humidified incubator in 5% CO\(_2\) at 37 °C.

Reverse phase high performance liquid chromatography (RP-HPLC) of phenols—Procedure of RP-HPLC: Quantitative fractionation of various phenolic acids in the methanolic samples was studied by RP-HPLC analysis. A modified method of Beta \textit{et al.}\(^{10}\) was followed for HPLC analysis. An HPLC system (Waters Associates) equipped with a 7725 Rheodyne injector and Waters 510 HPLC pump, 486 tunable absorbance detector and Millennium 2010 software data module was used. An elution period of 20 min with a flow rate of 0.8 mL min\(^{-1}\) was given. Sample (10 µL) was injected and the absorbance at 254 nm was recorded. Standard phenolic acids such as gallic, vanillic, \(p\)-hydroxybenzoic, ferulic, chlorogenic, sinapic, \(para\) coumarate and cinnamic acids were injected into the column separately. Comparing with the retention time of the standard phenolic acids and height of the peaks was taken for quantification.

Transmission electron microscopy analysis of MCF 7 cells—The cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylic buffer, pH 7.4 for 2 h at 4 °C, washed with 0.1 M cacodylic buffer, pH 7.4 for 12 h, and post fixed with 1% osmium tetroxide with 0.8% \(K_2[Fe(CN)]_6\) in cacodylic buffer for 1 h at room temperature. The material was dehydrated in an ethanol gradient (30, 50, 70, 80, 90, 96, and 100 %). The dehydrated pellets were embedded three times with propylene oxide for 1 h each and infiltrated with a resin/propylene oxide mixture at a 1:1 ratio for 2 h and then with resin only for 12 h at ambient temperature. The inclusion was made with Epon 812 and Araldite and the polymerization was performed at 60 °C for 48 h. Ultra thin sections were stained with uranyl acetate and counterstained with lead citrate\(^{11}\). Samples were examined and photographed in a JEOL 1200 EX electron microscope at 80 kV.

Cell proliferation assay—to determine the effect of methanol or water extract of \textit{L. bowringii} on MCF 7 cell proliferation, cells were seeded in 6-well culture plates at a concentration of \(6 \times 10^3\)well. Complete medium with 10, 25 or 50 µg/mL of lyophilized methanol or water extract was replaced every other day, beginning the day after seeding. Culture medium containing 0.1% DMSO was used as a solvent control. Cells cultured in complete medium were harvested with trypsin-EDTA on 5\(^{th}\) day after treatment and counted by a model Z1 Coulter particle counter (Coulter Corp, Miami, Florida).

Cell adhesion assay—Cell adhesion assays were performed using 12 well culture plates coated with 30 µg of Matrigel (Becton Dickinson, Bedford, MA). To block nonspecific binding sites, Matrigel coated culture plates were re-hydrated with serum free DMEM containing 0.1% BSA for 90 min at 37 °C and then washed with the same medium. MCF 7 cells were trypsinized and resuspended in serum free
DMEM containing 0.1% BSA. Cells were seeded at a concentration of $2 \times 10^5$ cells/well in presence of 0.1% DMSO or of 10, 25 or 50 µg/mL of *L. bowringii* methanol or water extract and incubated for 90 min at 37 °C and 5% CO$_2$. At the end of 90 min, the medium was removed and the cells were washed thoroughly twice with PBS to remove unattached cells. The attached cells were harvested by trypsinization, resuspended and counted. Each assay was performed in triplicate and repeated in three independent experiments. Values were expressed as the average of triplicate experiment

**Cell migration assay**—Scratch wound assay was used for mobility analysis. Cells were seeded into 6-well cell culture plates at a concentration of $3 \times 10^5$ cells and cultured in 10% FBS DMEM to near confluence. The confluent monolayer was carefully wounded using a sterile pipette tip and cellular debris was washed gently with PBS. The wounded monolayer was incubated in 10% FBS DMEM containing 20 µg/mL fibronectin and DMSO (0.1%) or 10, 25 or 50 µg/mL of methanol or water extract for 24 h. Migrating cells were examined and photographed under 10 x by phase contrast microscope.

**Invasion assay**—Cell migration across a Matrigel barrier (invasion) was determined by a modified Boyden chamber. Briefly, Boyden chambers were assembled using 8 µm Falcon transwell inserts (Becton Dickson, Bedford, MA) as the upper chamber and 12-well plates as the lower chamber. Matrigel was applied to the insert and kept overnight to dry in a laminar flow fume hood. The insert was re-hydrated with serum free DMEM for 90 min at 37 °C and 5% CO$_2$. Following rehydration, Matrigel coated inserts were washed with serum free DMEM. Exponentially growing breast tumor cells were harvested by trypsin/EDTA and resuspended in 0.1% BSA DMEM. Cells were seeded at a concentration of $2 \times 10^5$ cells. DMSO (0.1%) or 10, 25 or 50 µg/mL of methanol or water extract was carefully added to the upper chamber. Fibronectin (25 µg) was added as a chemo-attractant to the lower chamber. The Boyden chamber was incubated for 24 h at 37 °C and 5% CO$_2$. At the end of incubation, the cells in the upper chamber were removed by gently wiping with a cotton swab. Cells that transversed to the Matrigel and attached to the lower surface of the insert were fixed with 10% formalin and stained with 0.5% crystal violet. Inserts were examined under 20x or 40x phase contrast field microscopy and photographed. Values for invasion were expressed as the number of migrated cells per microscopic field. Four fields were counted. Each assay was performed in triplicate and repeated in three independent experiments. Values were expressed as the average of triplicate experiments.

**Cytotoxicity and apoptosis assay**—Cytotoxicity was assessed by a modified version of MTT reduction assay as per the methodology of Kumar and Karunagaran. For analyzing chromatin condensation by Hoechst 33342 staining, the cells were grown on 96-well plates and stained with 0.5 µg/mL of Hoechst 33342 for 10 min and viewed under UV filter sets using Nikon Epi-fluorescent microscope (TE2000E). Cells with apoptotic condensed nuclei were scored in percentage from 200 to 300 cells/sample at least by two investigators.

**Gelatin zymography**—MMP-2 and MMP-9 activity in the conditioned media was assayed by gelatin zymography. Briefly, MCF 7 cells were seeded at a concentration of $2 \times 10^5$/well in 6-well plates. After treatment with DMSO (0.1%) or 10, 25 or 50 µg/mL of methanol or water extract for 24 h, the supernatants were collected and subjected to gel electrophoresis on 10% running gels containing 0.1% gelatin. The gels were rehydrated with 10% formalin and stained with 0.5% Coomassie brilliant blue and then destained with destaining solution (10% v/v methanol and 5% v/v acetic acid), examined and photographed.

**Statistical analysis**—Sigma Stat software was used for statistical analysis. Results were expressed as the mean±SD. of three independent experiments. Comparisons were based on one-way ANOVA followed by Duncan's multiple range test. A *P*-value < 0.05 was considered statistically significant.

**Results**

RP-HPLC was performed to fractionate the phenols present in the methanolic extract of *L. bowringii*. The major phenolic acids recorded were (µg/g tissue) sinapate (87.37), ferulate (34.83), vanillate (11.27), chlorogenate (11.13), catechol (13.28), cinnamate (11.08), coumarate (113.98), and gallate (10.42). The high and diverse phenolic acids in the plant, suggest their role as precursor of many of the cellular
secondary metabolites. The presence of cinnamate, coumarate, gallate, ferulate has potential antioxidant property, which in turn suggests the antioxidant significance of the plant. Chlorogenic acid can regenerate oxidized vitamin E via caffeic acid and it also acts as a pro-oxidant in the propagation phase of low density lipoprotein oxidation\textsuperscript{14}. Coumarate is a precursor of flavonoids and also binds with nitric acid and its derivatives, before they combine with protein amines to form nitrosamine. Similarly cinnamate has antibacterial, antifungal and antiparasitic properties\textsuperscript{15}. Gallic acid, the well known natural antioxidant found widely in plants, and its derivative exhibited higher free radical scavenging activities than ascorbate.

**Ultrastructural analysis of methanolic extract of *L. bowringii* treated MCF 7 cells**—The cells revealed typical morphological features of apoptosis like cell shrinkage, plasma membrane blebbing, condensation of cytoplasm and chromatin, fragmentation of the rough endoplasmic reticulum (RER), loss of ribosomes, and changes in mitochondrial ultrastructure compared to control cells (Fig. 1a). Cells sharing morphological features like apoptosis and autophagy (Fig. 1b, c, d) were also observed, indicating the possibility of

---

**Fig. 1**—Electron microscopic photograph showing ultrastructural features of MCF 7 cells exposed to methanolic extract of *L. bowringii* (25 µM) for 6h [(a) control cell, (b) coexistence of morphological features of apoptosis and autophagy, (c) fragmentation of mitochondria, (d) autophagic vacuoles). Bar = 1 µm].

---
coexistence of apoptosis and autophagy of PCD types. The cells exhibited condensation and marginalization of chromatin that is characteristic of apoptosis, but also typical characteristics of autophagy-autophagic double-membraned giant autophagolysosomes containing cytoplasmic fragments and organelles like: RER, mitochondria, dense bodies, lysosomes and ribosomes (Fig. 1b, c). At more advanced stages, the autophagic vacuoles contained disintegrated cellular structures. Among such cells, cells with typical features of autophagy like: nuclei with centrally condensed chromatin, well-developed golgi complex, heavily vacuolized cytoplasm, with a few short channels of RER, and abundant free ribosomes were present (Fig. 1 d).

**Effect of extracts of L. bowringii on proliferation of MCF 7 cells**—The methanolic extract inhibited the proliferation of MCF 7 cells at all concentrations and was more significant than water extract (Fig. 2). Water extract significantly inhibited proliferation at the higher concentrations i.e. 25 and 50 µg/mL, but not at 10 µg/mL. At concentration 100 µg/mL or above, the number of cells on the 5th day after treatment was decreased to less than $6 \times 10^4$/well, compared to the number of cells seeded at the beginning of proliferation assay. However, extracts at the 10, 25, and 50 µg/mL concentrations inhibited the cell growth and no cytotoxicity was observed. Thus, for further studies of antimetastatic effect, extracts at the concentrations of 10, 25 and 50 µg/L were used.

**Effect of extracts of L. bowringii on the adhesion of MCF 7 cells**—The extracts remarkably inhibited the adhesion of cancer cells. Methanolic extracts down-regulated the binding of the cells to Matrigel and significantly inhibited adhesion of MCF 7 cells in a concentration dependant manner (Fig. 3). At a concentration of 50 µg/mL, adhesion was reduced by 50% compared to the control. Water also had a significant inhibitory effect on adhesion only at a concentration of 50 µg/mL.

**Effect of extracts of L. bowringii on the migration of MCF 7 cells**—The effect of extracts on MCF 7 cell migration was analyzed using the scratch wound assay. In absence of extract, MCF 7 cells migrated along the edges of the wound and covered the wound. Significant inhibition of cell flattening and spreading was observed in presence of methanolic and water extracts (Fig. 4).

**Effect of extracts of L. bowringii on the invasion of breast cancer cells**—Boyden chamber model was employed in the analysis of invasion of MCF 7 cells. Both methanolic and water extracts showed a concentration dependent suppression of the invasion of MCF 7 cells (Fig. 5 A and B). The methanolic extract inhibited the invasion of breast cancer cells more effectively than water extract.

**Cytotoxicity and apoptosis**—Chromatin undergoes condensation subsequent to cleavage of structural element of nuclease by activated caspases. In order to understand the nature of cell death induced by methanolic and water extracts, chromatin condensation was analyzed in cells treated with different concentrations of extracts, followed by staining cells with cell permeable DNA-specific stain, Hoechst 33342. The cells with intense localized fluorescence in the nucleus was scored as condensed and apoptotic. A representative image of cells stained for chromatin condensation is shown in Fig. 6.

![Fig. 2—Effect of extracts of L. bowringii on MCF 7 cell proliferation [Values are mean±SD. $P<0.05$].](image1)

![Fig. 3—Adhesion assay of MCF 7 cells in presence or absence of L. bowringii extracts [Values are mean±SD. $P<0.05$].](image2)
About 51% cells showed condensed nuclear morphology, with 50 µg methanolic extract after 24 h compared to 25% in water extract. Condensed nuclei (96% and 72%) were observed at 100 µg methanolic and water extracts respectively. The results from these experiments suggest that methanol extracts induced massive chromatin condensation even at low concentration compared to water extracts.

Effect of extracts of L. bowringii on MMP-2 and MMP-9 enzyme activities—The activities of gelatinolytic MMP-2 and MMP-9 in MCF 7 breast cancer cells was analyzed to determine whether the anti-invasive activity of extract was correlated with the inhibition of MMP activity (Fig. 7). Methanolic extract had an inhibitory effect on the MMP-9 activity in a dose dependent manner, whereas, MMP-2 activity only at the concentration of 50 µg/mL. Water extract also showed an inhibitory effect more on the MMP-9 activity.

Discussion

Breast cancer is an epithelial tumor with high invasive and metastatic potential. Despite major advances in early detection and adjuvant therapy, advanced breast cancer remains a major clinical problem. Therefore, there is an urgent need for new therapeutic approaches. Breast cancer invasion is a multiple step process characterized by altered cellular adhesion, increased motility and invasion of the extracellular matrix. In the present study, the anticancer effects of methanolic and water extracts of L. bowringii have been investigated using proliferation, adhesion, migration, invasion and matrix metalloproteinase activities in MCF 7 cancer cells.

Cells treated with methanol extract inhibited the proliferation of MCF 7 cells more effectively than water extract (Fig. 2), thus confirming the ethnobotanical knowledge of anti-tumor promoting activity of the plant. The results are significant than ether extract of Scapania verrucosa, a bryophyte on tumor cell lines. The phenolic acids in the extract of L. bowringii may combat biologically aggressive cancers, including metastasizing cancers, through targeting of specific kinases. Similarly, the polyphenols inhibit matrix metalloproteinases (MMPs) which are intimately associated with tumor invasion and metastasis.

The antiproliferative effect was also evidenced by the decrease of the density of microvilli that play an important role in the mitogenesis of MCF 7 cells. Further, various other ultrastructural features such as decrease of the golgi complex and the highly condensed heterochromatin are associated with low protein synthesis, the vesiculation of the rough endoplasmic reticulum is associated with an influx of water and sodium into the cell (Fig. 1). All these findings represent distinctive features of a damaged cell system. Moreover, the extracts induced mitochondrial swelling with a dense matrix in comparison to control MCF 7 cells, which presented globular mitochondrial with regular lamellar cristae. In breast cancer cells, it can be supposed that, besides the implication of mitochondria in phenomena of oxidative stress the morphological alterations of membranes being a possible consequence of variations in transcription and consequently, transduction. The presented results strongly suggest that autophagy is
not the only alternative but can be complementary to apoptosis under the influence of extract.

Many plant-derived molecules have inhibitory effects on tumor cell invasion, primarily via suppression of the activity of extracellular matrix (ECM). A prominent inhibitory effect of extract of *L. bowringii* on cancer cell attachment ability was also observed in the present study. The inhibitory effect of extract may be due to suppression of adhesion protein expression and/or the activity of adhesion molecules. The present findings indicate that the extract may inhibit signaling pathways involved in regulating the E-cadherin/catenin complex and possibly other cell–cell adhesion genes via β-catenin alteration.

Another key factor for cancer cell invasion and metastasis is cell migration. The effect of extract on MCF 7 cell migration using the scratch wound assay, revealed an inhibitory effect on cancer cell migration. The methanol and water extracts suppressed MCF 7 cell migration compared to the control (Fig. 4). Antiproliferative properties of the extract can contribute to inhibitory effect on the migration (Fig. 5). However, in the present study, water extract was less effective on the inhibition of cell proliferation than methanol extract, whereas the
inhibitory effects of methanol and water extracts on cell migration were similar. Azios and Dharmawardhane\textsuperscript{22} reported that resveratrol, a phytoestrogen present in grape skin and red wine, inhibited breast cancer cell migration by its antiproliferative and proapoptotic properties. Similarly, other mechanisms which can regulate cell migration need to be investigated to describe the effect of \textit{L. bowringii} extracts on cell proliferation and migration.

A crucial event in breast cancer invasion and metastasis is the invasion of cancer cells through the extracellular matrix\textsuperscript{23}. In the present study, in the Boyden chamber model, both methanolic and water extracts dramatically suppressed the invasion of MCF 7 cells (Fig. 5a). The methanol extract inhibited the invasion of breast cancer cells more effectively than water extract. To successfully penetrate the Boyden chamber, cells must successfully adhere, degrade and transverse the Matrigel coated insert\textsuperscript{24}. Therefore, in
the present study, the inhibitory effect of invasion could be explained by its inhibition of adhesion and migration. DNA damage and other forms of stress often induce p53 dependant cell cycle arrest or apoptosis. The molecular switch that governs the choice between cell-cycle arrest and apoptosis is not clear at present.

Matrix metalloproteinases (MMPs) are structural and functional related endopeptidases and plays crucial role in tumor invasion and building of metastatic formations because of their ability to degrade extracellular matrix proteins. Under physiological conditions, their activity is precisely regulated in order to prevent tissue disruption. This physiological balance seems to be disrupted in cancer making tumor cells capable of invading the tissue. To determine whether the anti-invasive activity of extract was correlated with the inhibition of gelatinolytic activities of MMP-2 and MMP-9 in MCF 7 breast cancer cells was analyzed (Fig. 7). Both methanolic and water extracts significantly inhibited MMP-9 than MMP-2 activities. Therefore, inhibitory effects of extract on the activities of MMPs may exert to the inhibition of invasion and MMP-9 is more important than MMP-2 for the inhibition of invasion. Methanol extract was more effective on the inhibition of activities of MMPs than water extract and this inhibitory effect was correlated with inhibition of invasion in the MCF 7 cells. The findings about the high expression of MMP-9 in the specimens analyzed are in accordance with data published by Przybylowska et al., who found increased levels of MMP-9 to correlate with G-3 breast cancer.

Overall, the present study substantiates that, the extract of L. bowringi has potential anticancer property by suppressing invasiveness of MCF 7 breast cancer cells. The cellular basis for this effect rests on the inhibition of MMP activities, which are required for cell migration as initial steps in the multistep process of tumor metastasis. Therefore, these MMPs are possible candidates for further in vitro functional analysis of their role in breast cancer biology.

Acknowledgement
The authors acknowledge Kerala State Council for Science Technology and Environment (KSCSTE), Govt. of Kerala for funding the project.

References
13. Kumar R R & Karunagaran S D, Ecotopic expression of Bcl-XL or Ku70 protects human colon cancer cells (SW480) against curcumin induced apoptosis while their down regulation potentialities, Carcinogenesis, 25 (2004) 1867.


