Chapter 6

Evaluation of the inhibitory effect of selected plant isolates on the development and metastatic progression of mouse 4T1 breast tumour
6.1. Introduction

To win the battle against breast cancer we have to give prime importance for combating metastasis. Metastasis is the leading cause of breast cancer-associated deaths. 30-40% of patients may eventually suffer from distant relapse and succumb to the disease, even though there is a significant improvement in current therapies in extending patient life. Deeper understanding of the metastasis biology is very important in developing better treatment strategies and achieving long-lasting therapeutic efficacies against breast cancer (Jin and Mu, 2015). To study metastasis the \textit{in vivo} metastatic models that mimic human breast cancer metastasis are limited, when xenograft models are used the exact replication of the human metastasis is difficult and these models only represents the primary tumour growth (Bibby, 2004; Eccles et al., 1994; Hoffman, 1999). The syngenic mouse 4T1 breast tumour model metastasizes in mice with normal immune function. This type of immunocompetent models is necessary for the study of cancer therapeutics because immune system also play a significant role in tumour development and progression. 4T1 mouse breast tumour model has many advantages to be used as suitable animal model that represent human mammary carcinoma. First of all 4T1 primary tumour will develop on the anatomically correct site. Secondly metastasis will develop spontaneously from primary site. Finally the pattern of metastatic spread is similar to the human condition (Pulaski and Rosenberg, 2001).

In order for its departure from primary site and to recolonise in different organs cancer cells need to undergo a series of changes. This metastasis cascade (Nguyen et al., 2009) comprises: cancer cells becoming locally invasive and migratory, reaching the blood vessel and intravasating into the circulation, circulating via the blood flow, arresting and extravasating to the distant organ, surviving the initial hostile stress, and reinitiating outgrowth and co-opting the distant stroma. Thus, it is more and more recognized that survival and outgrowth in foreign tissue are the rate-limiting steps for metastasis colonization and constitute the bottleneck of the metastasis cascade (Vanharanta and Massague, 2013). In the present study we analyzed the effect of some selected plant isolates on the organ specific tumour progression in a drastic breast cancer metastatic
condition in murine 4T1 model. Instead of going for a blind selection of test materials, we evaluated the plant products with strong literature background on pulmonary antimetastatic effects in the spontaneous metastatic B16F10 mouse model, punarnavine (Manu and Kuttan, 2009), Harmine (Hamsa and Kuttan, 2011) and an active fraction from *Emilia sonchifolia* (George and Kuttan, 2016; Gilcy and Kuttan, 2016), enriched with γ-humulene (71%) (γ-hum).

**6.2. Materials and Methods**

**Plant materials:**-
- Punarnavine an alkaloid from *Boerhaavia diffusa*
- Harmine β-Carboline alkaloid from *Peganum harmala*
- An active fraction from *Emilia sonchifolia*, enriched with the sesquiterpene γ-humulene (71%) (γ-hum).

**Dosage:**-

- Punarnavine - 40mg/kg body weight
- Harmine - 10mg/kg body weight
- γ-hum - 5mg/kg body weight

Administration - Intraperitoneal (i.p) for ten consecutive days.

**Animals:** - Female BALB/c mice (8 weeks old)

**Cell line:** - 4T1 mouse breast carcinoma cell line

Site of injection subcutaneous to the abdominal mammary fat pad

**6.2.1. Tumour volume and rate of survival**

Mice were divided into four groups (22 animals/group). All animals were injected with a cell suspension of 0.1 ml, containing $7 \times 10^4$ 4T1 cells into the fat pad of the 4th mammary gland. Group I was kept as the untreated tumour control. Group II animals were treated with punarnavine 40mg/kg body weight.
Group III animals were treated with harmine 10mg/kg body weight. Group IV animals were treated with γ-hum 5mg/kg body weight. The administration of the test materials were done intraperitoneally for 10 days starting from 7th day of tumour induction. On every 7th day solid tumour development was measured with vernier calipers and the tumour volume was calculated using the formula

\[ V = \frac{LW^2}{2} \]  

(V, tumour volume; L, length; W, width).

The remaining 14 animals in each group were observed for their survival.

6.2.2. Organ specific metastatic tumour progression

Mice were divided into four groups. All animals were injected with a cell suspension of 0.1 ml, containing \(7 \times 10^4\) 4T1 cells into the fat pad of the 4th mammary gland. Group I was kept as the untreated tumour control. Group II animals were treated with punarnavine 40mg/kg body weight. Group III animals were treated with harmine 10mg/kg body weight. Group IV animals were treated with γ-hum 5mg/kg body weight. Administration of the test materials were done intraperitoneally for 10 days starting from 7th day of tumour induction. Animals were sacrificed on particular time point’s, organs were collected and metastatic cell density was assessed. For histopathological analysis specific organs from each group of sacrificed animals on the particular day of organ collection were fixed in 10% formalin, dehydrated in different concentrations of alcohol and embedded in paraffin wax. Sections were stained with eosin and hematoxylin (Pulaski and Rosenberg, 2001).

6.2.2.1. Metastatic tumour progression to lymphnode

On the 18th day of tumour inoculation eight animals from each group were sacrificed. The draining lymphnodes were collected and placed in a tissue culture dish containing 10ml culture medium (DMEM) supplemented with 60µM 6-thioguanine. The samples were dissociated by forcing through a cell strainer. The cells were evenly distributed by swirling the dish. The dissociated cells were then incubated in 37°C with 5% CO₂. When the cells were attached
medium with unattached cells were removed. Cell density was assessed and photographed (Pulaski and Rosenberg, 2001).

6.2.2.2 Metastatic tumour progression to lungs

On the 25th day of tumour inoculation sixteen animals from each group were sacrificed. Lungs from eight animals were used for assessing the tumour progression to lungs. Lungs and serum collected from the remaining 8 animals were used to study the biochemical parameters. To assess the metastatic tumour progression, collected lungs were placed in a tissue culture dish containing 5ml 1x HBSS. Using forceps the lungs were thoroughly cleaned and transferred to another tissue culture dish. With curved scissors the lungs were minced into small pieces. The minced tissue was then transferred into a 15ml conical tube containing 2.5ml collagenase type IV cocktail. Tissue culture plates were again washed with 2.5ml 1xHBSS and transferred to the same 15ml conical tube to a final volume of 5ml. The samples were placed at 4°C for 75 min on a shaker. After completion of enzyme digestion volume of the sample was made upto 10ml with 1xHBSS. Samples were filtered through a cell strainer to remove large chunks of undigested tissue. Samples were then centrifuged, supernatant was discarded and the pellet was resuspended in 10ml medium supplemented with 60µM 6-thioguanine. The resuspended cells were plated in a tissue culture dish and were evenly distributed by swirling the dish. The plates were then placed in 37°C, 5% CO₂ incubator. When the cells were attached medium with unattached cells were removed. Cell density was assessed and photographed (Pulaski and Rosenberg, 2001).

6.2.2.2.1. Lung collagen hydroxyproline content

The lungs were homogenized and lung collagen hydroxyproline estimation was done by the chloramine-T method with hydroxyproline as standard (Bergman and Loxley, 1970).
6.2.2.2. Lung hexosamine content

Lyophilized tissue samples were hydrolysed and evaporated to dryness. Hexosamine was estimated in the presence of Ehrlich’s reagent with glucosamine as standard (Elson and Morgan, 1933).

6.2.2.3. Lung uronic acid content

Uronic acid level was estimated in the presence of carbazole reagent with glucuronic acid lactone as standard (Bitter and Muir, 1962).

6.2.2.4. Serum sialic acid and γ-glutamyl transferase levels

Blood was collected by heart puncture, serum separated, and used to estimate serum sialic acid by thiobarbituric acid assay (Skoza and Mohos, 1976), with N-acetyl neuraminic acid as standard. γ-glutamyl transferase level in the serum was estimated by measuring the release of p-nitroaniline from γ-glutamyl p-nitroanilide in the presence of an acceptor (glycylglycine) using a p-nitroaniline standard (Szasz, 1976).

6.2.2.3 Metastatic tumour progression to liver

On the 32nd day of tumour inoculation eight animals from each group was sacrificed. The liver was collected and placed in tissue culture dish containing 5ml 1x HBSS. Using forceps the liver was thoroughly cleaned and transferred to another tissue culture dish. Liver was minced into small pieces using curved scissors. The minced tissue was then transferred into a 15ml conical tube containing 2.5ml collagenase type I cocktail. Tissue culture plates were again washed with hyaluronidase cocktail and transferred again to the tube to a final volume of 5ml. Samples were placed at 37°C for 20 to 30min on a shaker. After completion of enzyme digestion volume of the sample was made upto 10ml with 1xHBSS. Samples were filtered through a cell strainer to remove large chunks of undigested tissue. Samples were then centrifuged, supernatant was discarded and the pellet was resuspended in 10ml medium supplemented with 60µM 6-
thioguanine. Samples were plated in a tissue culture dish and were evenly distributed by swirling the dish. The plates were placed in a 37°C, 5% CO₂ incubator. When the cells were attached medium with unattached cells were removed. Cell density was assessed and photographed (Pulaski and Rosenberg, 2001).

6.2.3. Determination of serum VEGF, IL-1β, TNF-α and GM-CSF levels

Mice were divided into two groups (8 animals/group). All animals were injected with a cell suspension of 0.1 ml, containing approximately $7 \times 10^4$ 4T1 cells into the fat pad of the 4th mammary gland. Group I was kept as the untreated tumour control. Group II animals were treated with punarnavine 40mg/kg body weight. Blood was collected from the caudal vein of all the experimental animals on 7th and 42nd day of tumour induction. Serum was separated and used for the estimation of VEGF, IL-1β, TNF-α and GM-CSF using highly specific ELISA kits according to the manufacturer’s instructions.

6.2.4. Expression of genes involved in metastasis

Total RNA was isolated from primary tumour of the punarnavine 40mg/kg body weight treated animals as well as control animals on the 32nd day of tumour induction. cDNA was synthesized using Moloney murine leukaemia virus reverse transcriptase. Amplification was performed using specific primers of MMP-2, MMP-9, TIMP-1, TIMP-2 and VEGF. GAPDH was selected as a stable reference gene under the experimental conditions in this study.

6.3. Results

6.3.1. Effect of test materials on tumour volume and rate of survival

The effect of test materials on solid tumour reduction is presented in figure 6.1. Administration of punarnavine reduced the tumour volume in experimental animals compared to tumour control and tumour along with harmine and γ-hum treated groups. Mice carrying 4T1 cells alone (tumour controls) had a tumour
volume of 937±194 mm$^3$ on day 42nd; it was reduced by the administration of punarnavine on the same day (408±139 mm$^3$). When tumour-bearing mice were treated with harmine and γ-hum the tumour volume was reduced to 892±201 mm$^3$ and 886±143 mm$^3$ on the same day. The data shows that there was significant reduction in tumour volume ($p<0.001$) in punarnavine treated group compared to the non significant reduction in tumour volume in harmine and γ-hum treated groups. The survival plot as shown in figure 6.2 also shows that the survival rate of animals treated with punarnavine was very high compared to the early death pattern shown by the tumour control, harmine and γ-hum treated groups.

6.3.2. Effect of test materials on metastatic tumour progression to lymphnode

Figure 6.3. gives a detailed picture of metastatic 4T1 cells present in the lymph node. The histopathology as well as the fold decrease in metastatic cell density clearly shows the reduction in the metastatic cells in the punarnavine treated group compared to tumour control, harmine and γ-hum treated groups.

6.3.3. Effect of test materials on metastatic tumour progression to lung

Figure 6.4. shows metastatic 4T1 cells present in the lung. The fold decrease in metastatic cell density in punarnavine treated group was very high compared to control and other two test materials treated groups. The histopathological analysis of the lung tissue was also confirmed the effect of punarnavine on the inhibition of metastatic progression compared to other groups.

6.3.4. Effect of test materials on metastatic tumour progression to liver

Liver metastasis also was significantly reduced by punarnavine treatment as shown in figure 6.5. Histopathological comparison of liver tissue and the metastatic cell density analysis in the four groups of animals also confirmed the effect of punarnavine in blocking the tumour progression in 4T1 induced animals compared to the other three groups of mice.
Figure 6.1. Solid tumour volume

Figure 6.2. Rate of survival
Figure 6.3. Metastatic progression to lymph node

Histopathology

Metastasized cells after 6-thioguanine treatment

A. Tumour control
B. Punarnavine
C. Harmine
D. γ-hum

Metastasized cell density in fold change
Figure 6.4. Metastatic progression to lung

Histopathology

A  B  C  D

Metastasized cells after 6-thioguanine treatment

A. Tumour control
B. Punarnavine
C. Harmine
D. γ-hum

Metastazised cell density in fold change

![Fold change graph]
Figure 6.5. Metastatic progression to liver
Histopathology

Metastasized cells after 6-thioguanine treatment

A. Tumour control
B. Punarnavine
C. Harmine
D. γ-hum

Metastasized cell density in fold change

Figure 6.7. Effect of punarnavine on gene expression

C- Control; T-Punarnavine
6.3.5. Effect of test materials on biochemical parameters

Elevated levels of lung hydroxyproline, hexosamine and uronic acid were shown by 4T1 induced tumour bearing mice. By the administration of punarnavine this levels were significantly (p<0.001) reduced in punarnavine treated group. This reduction was not significant in the other two harmine and γ-hum treated groups. The serum sialic acid and GGT level also showed a similar pattern of reduction as above in the punarnavine (p<0.001) treated groups compared to the other three groups (table 6.1).

6.3.6. Effect of punarnavine on serum VEGF, IL-1β, TNF-α and GM-CSF levels

The effect punarnavine on the serum VEGF, IL-1β, TNF-α and GM-CSF levels confirmed its regulatory effect on these mediators. The VEGF level in the tumour bearing control group was 512±23pg/ml and GM-CSF level was 72±6pg/ml. IL-1β and TNF-α levels were 182±12pg/ml and 543±12pg/ml in the tumour control groups on the 42nd day. These levels were significantly reduced in punarnavine treated groups VEGF (296±25pg/ml), GM-CSF (34±3pg/ml), IL-1β (78±9pg/ml) and TNF-α (318±14pg/ml) as shown in figure 6.6.

6.3.7. Expression of genes involved in metastasis

The mRNA expression of prometastatic genes such as MMP-2, MMP-9 and VEGF was found to be upregulated in the metastatic-tumour bearing control animals. The expression of these genes was down-regulated or inhibited in mice treated with punarnavine. It was interesting to note that mRNA expression of antimetastatic genes TIMP-1 and TIMP-2 were absent or down regulated in metastatic tumour bearing control animals and the punarnavine treatment could increase its expression in the treated group of animals (Figure 6.7).
Figure 6.6. Effect of punarnavine on cytokine levels

A. TNF-α; B. IL-1β; C. GMCSF; D. VEGF

*p<0.001
### Table 6.1. Biochemical parameters

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hydroxyproline (µg/mg protein)</th>
<th>Uronic acid (µg/100mg tissue wet weight)</th>
<th>Hexosamine (µg/100mg tissue dry weight)</th>
</tr>
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<tbody>
<tr>
<td>Tumour Control</td>
<td>24.55±3.04</td>
<td>339.1±37.24</td>
<td>4.6±0.86</td>
</tr>
<tr>
<td>Punarnavine</td>
<td>9.9±1.21***</td>
<td>109±13.6***</td>
<td>1.7±0.42***</td>
</tr>
<tr>
<td>Harmine</td>
<td>25.2±2.8</td>
<td>347.11±36.81</td>
<td>5.1±1.2</td>
</tr>
<tr>
<td>γ-hum</td>
<td>25.5±2.9</td>
<td>348.2±37.5</td>
<td>5.05±1.1</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sialic acid (µg/ml serum)</th>
<th>GGT (nmol/ml serum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumour Control</td>
<td>128.67±14.13</td>
<td>121.8±11.9</td>
</tr>
<tr>
<td>Punarnavine</td>
<td>56.6±11.8***</td>
<td>51.9±6.7***</td>
</tr>
<tr>
<td>Harmine</td>
<td>128.4±15.4</td>
<td>125.5±8.4</td>
</tr>
<tr>
<td>γ-hum</td>
<td>129.2±16.5</td>
<td>122.3±9.5</td>
</tr>
</tbody>
</table>

Values denote mean ± SD, ***p< 0.001
6.4. Discussion

In the present study we analysed the effect of three potent plant isolated test materials in a drastic metastatic condition established by the inoculation of 4T1 breast mammary carcinoma cells in mice. Initially the results on solid tumour reduction and rate of survival showed the potent effect of punarnavine on primary breast tumour growth compared to harmine and γ-hum. These results were further confirmed by monitoring the organ specific metastatic progression in the tumour bearing animals. The metastasized 6-thioguanine resistant cells of lymphnode, lung and liver collected on different days after sacrificing the animals when allowed to grow in culture the punarnavine treated group showed the maximum lower cell density. The histopathology of these organs was also in favour for the efficacy of punarnavine in combating metastatic progression in comparison to other test materials.

The assessment of biochemical parameters like hydroxyproline, hexosamine uronic acid, sialic acid and GGT were also undoubtably proved the dominating inhibitory effect of punarnavine on breast tumour metastasis. Hydroxyproline is posttranslationally produced from proline and forms a major component of collagen the main cellular matrix protein. The degradation of collagen in the metastatic tumour tissues will result in an elevated level of hydroxyproline (Phang et al., 2008). The acidic and basic modifications of monosaccharides found in the extracellular matrix yield uronic acids (glucuronic acid) and amino sugars (hexosamines) and they form a vital part in many structural polysaccharides and glycosaminoglycans (GAG) found in the ECM. Hexosamine found in the ECM serves as ground substratum for collagen synthesis (West et al., 1985). Elevated expression of sialoglycans in the circulation of tumour bearing animals correlate with tumour aggressiveness and ability to metastasize and invade surrounding tissues. Aberrant sialylation contribute to therapy resistance of cancer. Sialic acids promote tumorigenesis by facilitating escape from apoptosis, formation of metastasis, and resistance to therapy. Selective approaches interfering with sialic acid expression would therefore have great potential to counteract tumour growth and metastatic formation (Bull et al., 2014). Higher serum levels of GGT are associated with an
increased cancer risk. GGT is disregulated in malignant cells and this will produce reactive oxygen species resulting in more aggressive tumour formation (Fentiman, 2012).

Numerous studies have indicated that tumor cells exhibit an elevation in the constitutive production of proinflammatory cytokines such as TNF-α, IL-1β, and GM-CSF. TNF-α can act as endogenous tumour promoter involved in the tumour progression (Balkwill, 2006). IL-1 expression increases the tumour invasiveness and metastasis by enhancing the expression of adhesion molecules on endothelial and malignant cells into the circulation and their dissemination to remote tissues (Guo et al., 2012). Elevated serum levels of tumour promoting factor GM-CSF in cancer patients is considered as marker with high diagnostic sensitivity. GM-CSF will enhance invasive capacity of cancer cells via increased expression of matrix degrading proteins. It is also involved in enhancing the expression of MMPs and this was reversible by GM-CSF blocking (Gutschalk et al., 2013). Lowering of the serum levels of these cytokines indicate the potent effect of punarnavine on the inhibition of cytokine mediated signaling that play an important role in tumorigenesis.

MMPs are a family of zinc dependent endopeptidases and MMP mediated ECM degradation leads to cancer cell invasion and metastasis. MMP-9 is critical for the formation of metastatic niche due to its ability to liberate VEGF and thereby support angiogenesis. MMP-2 and MMP-9 enhance tumour cell migration and contribute to the establishment of metastasis–prone sites at tumour distant organs (Kessenbrock et al., 2010). MMPs are produced in the zymogen form and their activation and activities are regulated by TIMPs. The disruption of MMP-TIMP balance will result in tumour invasion and metastasis (Chirco et al., 2006). In the present study, punarnavine treatment downregulated the expression of MMPs at the same time upregulated the expression of TIMPs, indicating its regulatory effect on the inhibition of MMP. When MMPs are lacking or TIMPs are over produced, formation of new tumours decreases as evident from these results.
Autocrine VEGF signaling crucial in highly aggressive cancers is mediated by growth, survival, migration and invasion of cancer cells (Kohno and Pouyssegur, 2006). In this study, the expression of VEGF has been downregulated and as supporting evidence the serum VEGF level was also reduced by the treatment. These results show that punarnavine could inhibit all the pathways that link the MMPs and VEGF to tumour survival, proliferation, and invasion.