RESEARCH ARTICLE

Inhibition of Metastatic Lung Cancer in C57BL/6 Mice by Marine Mangrove *Rhizophora apiculata*

V Vinod Prabhu, C Guruvayoorappan*

Abstract

Metastasis is one of the hallmarks of malignant neoplasms and is the leading cause of death in many cancer patients. A major challenge in cancer treatment is to find better ways to specifically target tumor metastasis. In this study, the anti-metastatic potential of the methanolic extract of *Rhizophora apiculata* (*R.apiculata*) was evaluated using the B16F-10 melanoma induced lung metastasis model in C57BL/6 mice. Metastasis was induced in C57BL/6 mice by injecting highly metastatic B16F-10 melanoma cells through the lateral tail vein. Simultaneous treatment with *R.apiculata* extract (10 mg/kg b.wt (intraperitoneal) significantly ($p<0.01$) inhibited pulmonary tumor nodule formation (41.1 %) and also increased the life span (survival rate) 107.3 % of metastatic tumor bearing animals. The administration of *R.apiculata* extract significantly ($p<0.01$) reduced biochemical parameters such as lung collagen hydroxyproline, hexosamine, uronic acid content, serum nitric oxide (NO), γ-glutamyl transpeptidase (GGT) and sialic acid levels when compared to metastasis controls. These results correlated with lung histopathology analysis of *R.apiculata* extract treated mice showing reduction in lung metastasis and tumor masses. Taken together, our findings support that *R.apiculata* extract could be used as a potential anti-metastasis agent against lung cancer.

Keywords: *Rhizophora apiculata* - metastasis - lung metastasis - B16F-10 melanoma - pyrazole - nitric oxide

Asian Pacific J Cancer Prev, **14 (3)**, 1833-1840

Introduction

Cancer is a term used for disease in which abnormal cells proliferate without control and invade other tissues. Cancer disease is a leading cause of death worldwide, accounting for 7.6 million deaths in 2008 and could continue to rise 13.1 million deaths by year 2030 (WHO, 2008). The main reason for such high mortality from cancer is due to the highly invasive behavior of cancer cells, which usually results in metastasis (Khan and Mukhtar, 2010). Metastasis is a process by which cancer cells spread to other parts of the body through blood circulation and lymphatic system. A tumor formed by metastatic cancer cells is called as metastatic tumor (Klein, 2008). Many metastasis tumor develops at the first area of blood vessels that cancer cells arrive after leaving the primary tumor. After leaving the primary tumor, the lungs are one of the first site where metastatic cells are carried by the bloodstream (Hyoudou et al., 2004). The other common site for the cancer cells to metastasize includes brain, bones, liver, adrenal gland, lymph nodes, peritoneum, skin and other organs.

Metastasis is an extremely complex process that remains to be a major problem in the management of cancer (Hunter et al., 2008). The metastatic process involves tumor cell invasion from the primary tumor, intravasation, arrest and extravasation of the circulatory system to form small tumors known as micrometastases that stimulate angiogenesis. Tumor cells break away from the primary tumor site and degrade proteins that make up the surrounding extracellular matrix (ECM) that separates tumor from neighboring tissues. Cancer cells degrades the protein, breach the ECM and metastasize to form secondary tumor at distant organs (Nguyen and Massagué, 2007). Metastatic cancer cells generally identical as cells of the primary cancer i.e. breast cancer that spreads to the lungs and forms a metastatic tumor is known as metastatic breast cancer, not lung cancer (Talmadge and Fidler, 2010). However, metastatic cancer cells and cells of the primary cancer usually have some molecular features in common such as the expression of certain proteins or the presence of specific chromosome changes. The competence of metastasize cells depends on the host's immune cells at the niche, blood circulation and capillary beds, but most of the cancer cells are been trapped by these barriers, but only few cancer cells prevail over these barriers and metastasize. Some time metastasized cancer cells can be dormant at distance niche for many years and could redevelop in later stage (Luzzi et al., 1998; Aragon Ching and Zujewski, 2007). More over there are many

*Department of Biotechnology, Karunya University, Karunya Nagar, Coimbatore - 641 114, Tamil Nadu, India *For correspondence: immunologykarunya@gmail.com, gurukarunya@gmail.com*

molecules involved during metastasis which includes adhesion molecules, proteases, cell mobility, ECM, growth factors, oncogenes, signal transductions and transcription factors.

Despite advancement in early cancer diagnosis and treatment included surgery, chemotherapy, radiotherapy and adjuvant therapies. Around 90% of cancer deaths are caused by metastasis that are resistant to conventional therapies (Gupta and Massague, 2006). Although there are several drugs that are used for cancer therapy, however there are no drugs available at present that blockade any single step in the metastatic process.

Natural products and their derivatives contribute more than 50% of all the drugs in clinical use of the world. Higher plants contribute not less than 25% of the total. Almost 60% of drugs approved for cancer treatment are of natural origin (Sithranga, 2010). Many experimental studies and clinical trial showed that many natural plants played an important role in blocking of lung metastasis from primary tumors (Schantz et al., 1987; Leyon and Kuttan, 2004; Leyon et al., 2005; Thejass and Kuttan, 2006).

Marine flora constitutes more than 90% of oceanic biomass that offer a great scope for discovery of new drugs. It is recognized that ocean contains a large number of natural products and novel chemical entities with unique biological activities that may be useful in finding the potential drugs with greater efficacy and specificity for the treatment of human diseases (Sithranga, 2010). The marine flora may contain novel compounds to withstand extreme variations in pressure, salinity, temperature and the chemicals produced are unique in diversity, structural, and functional features.

Mangroves have long been used in folk medicine to treat diseases and very few mangrove plants are reported for possible source of anticancer drugs, based on traditional knowledge and preliminary scientific work (Sithranga, 2010). Mangrove, *Rhizophora apiculata* (*R.apiculata*) family of Rhizophoraceae is halophytic used as folk medicine, based to on the fact that use of its root, leaf or stem extracts to a greater extent imparts an inhibitory effect on the growth of bacterial, viral and fungal pathogens (Premanathan et al., 1999; Antony et al., 2011). Polysaccharide extracted from the leaf of *R.apiculata* reported to inhibit HIV-1 or HIV-2 strains in various cell cultures (Premanathan et al., 1999). *R.apiculata* extract have a high content of flavanoids, tannins, catachin, anthroquinone, pyroligneous acid and syringol.

Our earlier report of phytochemical analysis of methanolic extract of *R.apiculata* by GC/MS and LC/MS analysis shown the presence of pyrazole (alkaloid), ketone, thiazolidinediones and 4-pyrrolidinyl with a wide range of biological properties such as anti-tumor, anti-inflammatory, immunostimulatory and chemoprotectant activities (Prabhu and Guruvayoorappan, 2012; 2012a). Anti-metastasis activity of *R.apiculata* extract have not been reported elsewhere, to provide validity to the claims that *R.apiculata* extract has numerous polyphenol compounds with potential health benefits. In the present study we have evaluated the anti-metastatic activity of methanolic extract of *R.apiculata* in B16F-10 melanoma cell induced lung metastasis in C57BL/6 mice.

Materials and Methods

Plant collection

R.apiculata (Vernacular name - Surapunnai in Tamil), whole plant were collected from the Pichavaram mangrove forest located in Cuddalore District, Tamil Nadu on the southeast coast of India. Pichavaram is the second largest mangrove forest in the world.

Experimental animals

C57BL/6 mice (male 4-6-wk-old) weighing (20-25g) were kept in a pathogen free air controlled room maintained at 24°C with an ~50% relative humidity and 12-hr light/dark cycle, and provided ad libitum access to normal mice chow (Sai Feeds, Bangalore, India) and filtered water. All animal experiments were performed according to the rules and regulations of the Institutional Animal Ethics Committee of the Government of India.

Cell lines

B16F10 melanoma cells were obtained from the National Centre for Cell Sciences (Pune, India) and maintained in DMEM (Hi Media, Mumbai, India) containing 10% fetal bovine serum (FBS; Hi Media) and 1% antibiotic/antimycotic solution. All culture steps took place at 37°C in a 5% CO_2/95% air incubator.

Chemicals

Gum acacia was purchased from Hi-Media, Formaldehyde solution was procured from Universal Laboratories Pvt. Ltd. (Hyderabad, India). All chemicals used were analytical or reagent grade.

Methanolic extract preparation

R.apiculata samples collected from the Pichavaram mangrove forest (Tamil Nadu) were taxonomically identified and authenticated at the Department of Botany at M.E.S. Kalladi College (Mannarkkad, India), and a voucher specimen was deposited (Accession Number: Rhiz-018). The plant material was dried at 45°C and then powdered. Ten gram of the material was stirred overnight in 70% methanol (100 ml), and then centrifuged at 10,000 rpm (10 min, 4°C). The resultant supernatant was collected and the methanol removed by evaporation (yield of final product=12% [w/w]).

For use in the animal experiments outlined below, this final material was re-suspended fresh in 1% (w/v) gum acacia and then administered via intraperitoneal (i.p.) injection at a dose of 10 mg/kg body weight (b.wt) daily, for 10 consecutive days. The non toxic concentration of 10 mg/kg b.wt was selected based on the in vivo toxicity studies and in vitro MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Mokkhasmit et al., 1971; Kuttan et al., 1985; Cole 1986; Prabhu and Guruvayoorappan, 2012).

Biochemical measurements

C57BL/6 mice were separated into two groups (9

nos/group) for the experiment. All the animals in two groups were induced metastasis by injecting metastatic B16F10 melanoma cells (1x10^{6}cells/animal) via lateral tail vein. Group I was kept as metastasis induced untreated control and received only vehicle. Group II were treated with *R.apiculata* extract (10 mg/kg b.wt. (i.p.) for ten consecutive days. The animals were euthanized and blood samples were collected from the animals on different day intervals (7th 11th and 21st day) serum samples were isolated for biochemical measurements such as NO, GGT and sialic acid levels.

Determination of the effect of R.apiculata on serum NO level of metastatic tumor bearing animals

On day (7th 11th and 21st day) serum NO level was estimated by the use of a Griess reaction. The reaction mixture containing sodium nitroprusside and serum was incubated at 25ºC for 150 min. After incubation, 1ml of the reaction mixture mixed with equal volume of Griess reagent and allowed to stand for 15 min at room temperature. The absorbance of pink colored chromophore formed was measured at 546 nm against the corresponding blank solutions and expressed in μm (Green et al., 1982).

Determination of the effect of R.apiculata on serum GGT level of metastatic tumor bearing animals

On day (7th 11th and 21st day) serum GGT level was estimated by measuring the release of p-nitroaniline from gamma glutamyl p-nitroaniline in the presence of glycyl glycine. The GGT content was determined from the graph plotted using p-nitroaniline as the standard (Tate and Meister, 1974).

Determination of the effect of R.apiculata on serum sialic acid level of metastatic tumor bearing animals

C57BL/6 mice were separated into two groups (9 nos/group) for the experiment. All the animals in two groups were induced metastasis by injecting metastatic B16F10 melanoma cells (1x10^{6}cells/animal) via lateral tail vein. Group I was kept as metastasis induced untreated control received only vehicle. Group II were treated with *R.apiculata* extract (i.p.) for ten consecutive days. Blood samples were collected by cardiac puncture on (Day 21) and serum was separated for the estimation of protein bound sialic acid level was determined by thiobarbituric acid assay (Skoza and Mohos, 1976; Bhavanandan et al., 1981). The serums were hydrolyzed using 0.2 N sulphuric acid. The hydrolysate was oxidized with periodic acid and incubated at 37ºC for 1 min. After terminating oxidation using sodium arsenate, 6% thiobarbituric acid was added. Sialic acid was estimated at 549 nm with reference to 532 nm after adding DMSO. Sialic acid content was determined from the standard graph plotted using n-acetyl neuraminic acid.

Determination of the effect of R.apiculata on lung collagen hydroxyproline, hexosamine, uronic acid and tumor nodules count of metastatic tumor bearing animals

C57BL/6 mice were separated into two groups (9 nos/group) for the experiment. All the animals in two groups were induced metastasis by injecting highly metastatic B16F10 melanoma cells (1x10^{6}cells/animal) via lateral tail vein. Group I was kept as metastasis induced untreated control received only vehicle. Group II were treated with *R.apiculata* extract (i.p.) for ten consecutive days. Animals from each group were euthanized on 21st days (final day of experiment) of tumor induction and the lungs were excised for counting tumor nodules and were used for the estimation of lung hydroxyproline (Bergman and Loxley, 1970), lung hexosamine (Elson and Morgan, 1933) and lung uronic acid (Bitter and Muir, 1962).

Determination of the effect R. apiculata on lung collagen hydroxyproline of metastatic tumor bearing animals

Lung collagen hydroxyproline was determined by the method of Bergman and Loxley (1970). The lungs were homogenized and protein precipitated with TCA were hydrolyzed for 24 hr at 110^{0}C in sealed glass tubes using 6 N HCl. The HCl was evaporated and the remaining hydrolysate residue was allowed to dryness. The residue was dissolved in distilled water and assay by chloramines -T method. The absorbance was measured at 560nm. Standard graph was plotted using reagent hydroxyproline standard.

Determination of the effect R. apiculata on lung hexosamine of metastatic tumor bearing animals

The hexosamine content present in the lung tissue was estimated by the method of Elson and Morgan (1933). Lyophilized tissue samples were hydrolyzed with 2 N HCl at 100^{0}C for 6 hr in sealed glass tubes. After hydrolysis, the HCl was evaporated and the remaining hydrolysate residue was allowed to dryness. The residue was dissolved in distilled water and treated with 2% acetyl acetone. The hexosamine level was determined in the presence of Ehrlich's reagent at 530 nm. Standard graph was plotted by using glucosamine standard.

Determination of the effect of R. apiculata on lung uronic acid content of metastatic tumor bearing animals

The uronic acid content present in the lungs of metastatic tumor bearing animals was estimated by the carbazole reactions (Bitter and Muir, 1962). The lungs were digested with crude papain and were hydrolyzed at 100^{0}C for 20 min in a sealed glass tubes. After hydrolysis, the hydrolysate was allowed to treat with sulfuric acid. The uronic acid level was determined by using carbazole reagent at 530 nm. Standard graph was plotted using by glucuronic acid lactone.

Determination of the effect of R. apiculata on the survival rate of metastatic tumor bearing animals

The remaining animals (n=3) of each group from the above experiment were observed for their survival rate. The mortality rate of each animal were observed and the percentage increase in life span (%ILS) was calculated using the formula %ILS=T-C/C×100. Where 'T' represents the number of survival days of treated animals and 'C' represents the number of survival days of control animals.

Histopathological examination

A portion of excised lung tissue were fixed in 10% formalin, cut into 5-μm thickness, stained using H&E (hematoxylin and eosin) and then examined for histopathological changes. The stained sections of lungs tissue samples were examined for large metastasis, hyperchromatic nucleus and pleomorphism.

Statistical analysis

The results are expressed as mean±SD. Statistical evaluation was performed using a one-way analysis of variance (ANOVA) followed by a Dunnett test using Graph Pad Instat (Version 3.0 for Windows 95; Graph Pad Software, San Diego, CA). p- values<0.05 were considered statistically significant.

Results

Effect of R.apiculata on the serum NO and GGT level of metastatic tumor bearing animals

The effect of *R.apiculata* on serum NO and GGT level in metastases tumor bearing animals is shown in Table 1. The administration of methanolic extract of *R.apiculata* significantly (p<0.01) reduced the serum NO level (27.20±0.31μm) on 21st day when compared with metastasis control (34.29±1.13μm) on the same day. The administration of *R.apiculata* extract also significantly (p<0.01) decrease the serum GGT level (44.30±1.4 nmol p-nitroaniline/ml) on 21st day when compared with metastasis control (81.44±4.0 nmol p-nitroaniline/ml) on the same day.

Effect of R.apiculata on serum sialic acid, lung hydroxyproline, hexoamine and uronic acid of metastases bearing animals

The effect of *R.apiculata* on serum sialic acid, lung hydroxyproline, hexoamine and uronic acid of metastases bearing animals is presented in Table 2. The administration of *R.apiculata* extract significantly (p<0.01) decreased the serum sialic acid level (35.51±0.42 μg/ml) when compared to metastasis control (105.33±0.91 μg/ml) on the same day. The *R.apiculata* extract significantly (p<0.01) reduced the lung hydroxyproline level (5.34± 0.17 μg/mg protein) when compared with metastatic control (20.25±1.12 μg/mg protein) on the same day.

The lung hexoamine level of *R.apiculata* extract treated animals were significantly (p<0.01) decreased (1.35±0.14 mg/100 mg tissue dry weight) when compared with metastatic control (3.17±0.17 mg/100 mg tissue dry weight). After the treatment of *R.apiculata* extract the lung uronic acid level significantly (p<0.01) reduced (93.20±2.30 μg/100 mg tissue wet weight) when compared with metastatic control animals (238.30±4.25 μg/100 mg tissue wet weight) on the same day.

Effect of R.apiculata on inhibition of lung metastatic nodule formation and on survival of animals

Effect of *R.apiculata* on inhibition of lung metastatic nodule formation and on survival of animals is shown in Table 3. B16F-10 melanoma cells which form lung tumor in experimental animals (Engbrin et al., 2002). The treatment with *R.apiculata* significantly (p<0.01) reduced (26.6±8.1) the number of countable colonies of pulmonary tumor nodules formation of B16F-10 melanoma cells in the lungs of the experimental mice when compared with metastasis control animals which shows massive growth of tumor nodules in lungs (45.2±9.0). The percentage of decrease in lung nodule formation of *R.apiculata* extract treated animals were (41.1%).

The survival rate of *R.apiculata* extract treated animals was increased when compared to metastatic control animals. The maximum survival rate were observed when *R.apiculata* extract administered (81±3.2) (107.6%) when

Table 1. Effect of *R.apiculata* on serum NO and GGT Level in Metastases Tumor Bearing Animals

Days	NO (μm)		
	7	11	21
Metastasis control	26.58±0.38	29.74±0.33	34.29±1.13
R. apiculata treated	25.83±0.31*	27.08±0.25**	27.20±0.31**
	GGT (nmol *p*-nitroaniline/ml)		
Metastasis control	9.64±0.6	29.71±1.7	81.44±4.0
R. apiculata treated	7.71±1.3	20.80±2.0**	44.30±1.4**

*Treatment animals received methanolic extract of *R. apiculata* (10mg/kg b.wt. (ip) for 10 consecutive days and metastatic control received metastatic B16F-10 melanoma cells (1x10^{6}cells/animal) via lateral tail vein and received only vehicle. Blood samples were collected by tail vein on 7th,11th and (cardiac puncture) 21st day. Serum samples were isolated to determine NO and GGT level. Value is significantly different from metastasis control (**p<0.01) (n=6). Values are expressed as mean±SD

Table 3. Effect of *R.apiculata* on Inhibition of Lung Metastatic Nodule Formation and on Survival of Animals

Treatment	No of tumor nodules	No of days survived
Metastasis control (C)	45.2±9.0	39±2.1
R.apiculata treated (T)	26.6±8.1**	81±3.2**

*Treatment animals received methanolic extract of *R. apiculata* (10mg/kg b.wt. (ip) for 10 consecutive days and metastatic control received metastatic B16F-10 melanoma cells (1x10^{6}cells/animal) via lateral tail vein and received only vehicle. On 21st day the animals were euthanized and lungs were dissected and lung metastatic nodules were counted. The percentage increase in life span is calculated by (T-C/Cx100), where T and C are the number of days survived by the treated animals and metastatic control animals respectively. Value is significantly different from metastasis control (**p<0.01). Values are expressed as mean±SD

Table 2. Effect of *R.apiculata* on Serum Sialic Acid, Lung Hydroxyproline, Hexoamine and Uronic Acid in Metastases Bearing Animals

Parameters	Serum Sialic acid (μg/ml)	Hydroxyproline (μg/mg protein)	Hexosamine (mg/100 mg tissue dry weight)	Uronic acid (μg/100 mg tissue wet weight)
Metastasis control	105.33±0.91	20.25 ± 1.12	3.17±0.17	238.30±4.25
R.apiculata treated	35.51 ± 0.42**	5.34±0.17**	1.35 ±0.14**	93.20±2.30**

*Treatment animals received methanolic extract of *R. apiculata* (10mg/kg b.wt. (ip) for 10 consecutive days and metastatic control received metastatic B16F-10 melanoma cells (1x10^{6}cells/animal) via lateral tail vein and received only vehicle. Blood samples were collected 21st day by cardiac puncture and serum samples were isolated to determine silaic acid level. On the same day lungs were dissected to determine the lung hydroxyproline, hexoamine and uronic acid level. Value is significantly different from metastasis control (**p<0.01) (n=6). Values are expressed as mean±SD

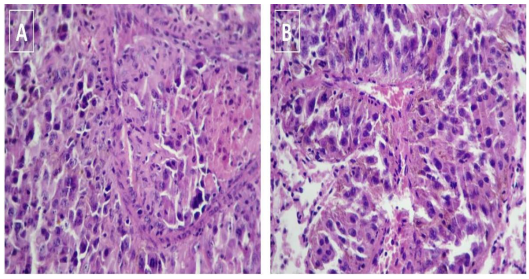

Figure 1. Histopathology of Lung Metastatic Tumor Bearing Animals. A portion of excised lung tissue from lung metastatic tumor bearing animals were fixed in 10% formalin, cut into 5-μm thickness, stained using H&E (hematoxylin and eosin) and then examined for histopathological changes. (A) Metastatic control animals shown prominent large metastasis with tumor nodules, hyperchromatic nucleus and necrosis. (B) Metastatic tumor bearing animals treated with *R.apiculata* extract shows reduction in the lung metastasis and tumor mass

compared to metastasis control animals (39±2.1) as shown in Table 3. The percentage increase in life span=T-C/Cx100, where T and C are the number of days survived by the treated and control group of animals respectively. The increase in survival days and increase in life span were ILS%=107.3%.

Histopathology analysis

Histopathology analysis of lung metastatic tumor bearing animals is shown in Figure 1. Pictures shown are from representative lung samples collected at the end of the experimental period (i.e., Day 21). (A) Metastatic control animals shown prominent large metastasis with tumor nodules and hyperchromatic nucleus and necrosis. (B) Metastatic tumor bearing animals treated with *R.apiculata* extract shows reduction in the lung metastasis and tumor mass.

Discussion

Metastasis is one of the hallmarks of malignant neoplasm or cancer which is the leading cause of death in many cancer patients (Nonaka et al., 1993; Lee et al., 2008). The degree of ability to spread varies between different types of tumors. In most cases, cancer patients with localized tumors have a better chance at survival than those with metastatic tumors. The metastatic capacity of a tumor cell is reliant on several factors. Tumor cells invade the tissues adjacent of primary tumor, increase their motility and migrated into the blood circulation (intravasate). Then the tumor cells elude the host defense mechanisms and adhere to a suitable niche. Consequently the tumor cells (extravasate) and invade into the secondary niche and evade apoptosis, regulates proliferation and angiogenesis (Chambers et al., 2001; Herzig and Christofori, 2002; Prabhu et al., 2012b). Among the metastasis cancers, the lung is the first organ to be encountered by the tumor cells making it a major site for tumor metastasis (Hyoudou et al., 2004). The survival rate for patients with metastatic melanoma is less than 10% (Bhatia et al., 2009). Tumor metastases are treated with surgery, radiotherapy, chemotherapy and hormone therapy or "multimodal therapy" (Wang et al., 2012). The metastatic treatment depends on, type of primary cancer, size and location of the metastases. Therefore, metastasis is a major target of cancer therapy and it is complicated to treat metastasis effectively with the available treatment with lesser survival rate (Bhatia et al., 2009). Many plants serve as novel drugs throughout the world. World Health Organization (WHO) reveals around 80% of the world population depends on plant derived medicine for their little of no adverse effects (WHO, 2008). Therefore in this study we have investigated the anti-metastatic potential of *R.apiculata* extract on B16F-10 metastatic lung cancer cell line in C57BL/6 mice.

Nitric oxide (NO) is a pleiotropic regulator with numerous biological processes, including vasodilatation, neurotransmission, macrophage-mediated immunity and immune defenses (Nathan, 1992). Concurrently NO also play an important role in tumor progression causing DNA damage, metastasis and angiogenesis (Shi et al., 1999; Fukumura and Kashiwagi, 2006; Prabhu and Guruvayoorappan, 2010). Increased NO generation in cancer cells has direct correlation with circulating tumor cells for their survival, high metastatic ability and angiogenesis by up-regulating vascular endothelial growth factor (VEGF) and VEGF-induced neovascularization (Edwards et al., 1996; Ambs et al., 1998). NO regulates some of the cellular adaptive responses to hypoxia which are associated with increased metastatic potential (Branco-Price et al., 2012). In the present study the *R.apiculata* extract significantly reduced the NO production level preventing high metastatic ability of tumor cells in experimental animals. The plant extract protective effect was further proved by showing reduction in lung tumor nodules (41.1%inhibition) compared to the metastatic control. This reduction in tumor nodules is correlated with an increase in the life span (107.6%) of the metastatic tumor bearing animals.

γ-glutamyl transpeptidase (GGT) is the only known enzyme that cleaves the g-glutamyl-cysteine peptide bond in GSH and other g-glutamyl compounds. GSH is synthesized intracellularly which provides energy to the tumor cells through gamma glutamyl cycle. GGT catalyzes glutathione (GSH) breakdown. An increase of GGT levels is a common finding in human tumors and as an important aspect of the tumor cell phenotype. In tumor cells, GGT over expression and its effects on GSH metabolism may affect several specific functions. It has been reported that variations in GGT expression and cellular levels of GSH is associated with modulation of metastatic properties of several tumors (Carretero et al., 1999). During tumor progression the serum GGT level increases due to cellular proliferation therefore serum GGT level can be used as marker for cellular proliferation (Pradeep and Kuttan, 2002). The administration of *R.apiculata* extract significantly reduced the serum GGT level in metastatic tumor bearing animals which is directly correlated with decrease in tumor cell proliferation and metastasis.

Sialic acid is a derivative of neuraminic acid occurs as a terminal component of carbohydrate chain of glycoproteins. Sialic acid at the terminal position is involved in cellular adhesion (components of many cell

surface receptors) and have ability to cellular recognition sites during invading foreign cells including cancer cells (Schauer, 1985). Metastatic cancer cells often express a high density of sialic acid-rich glycoproteins (Fuster et al., 2005). Total serum sialic acid level has been recognized as a valuable non-specific monitor of tumor burden in melanomas. The increase in the sialic acid level on the surface of malignant cells creates a negative charge on cell membrane which creates electrostatic repulsion between cells that initiates the malignant cells to metastasize through blood circulation (Fuster et al., 2005). The amount of sialic acid on the surface of malignant cells is directly correlated with the metastatic ability of the tumor cells (Vedolva and Borovansky, 1994). The treatment of *R.apiculata* extract significantly decreased the serum sialic acid level in metastatic tumor bearing animals. The *R.apiculata* extract prevented the expression of sialic acid on the surface of malignant cells preventing negative charge and electrostatic repulsion thus inhibiting metastasis. The decreased in serum sialic acid level were directly correlated with the reduction in metastasis.

Hydroxyproline is a major component of the protein collagen. Hydroxyproline and proline play key roles for collagen stability (Nelson and Cox, 2005). In the lung, collagen is found associated with bronchi, with blood vessels, and with the alveolar interstitium; it plays an important role in the maintenance of lung structure and function. During lung metastasis there will be enormous accumulation of fibrosis in lungs resulting in substantial deposit of collagen (hydroxyproline) in the alveoli of lungs (Pradeep and Kuttan, 2002). Elevated level of collagen in the lung is associated with pulmonary fibrosis resulting in inability of the lung to function normal gas exchange (Voet and Voet, 1995). Therefore lung hydroxyproline level has been used as an indicator to determine collagen amount and lung fibrosis (Voet and Voet, 1995). In the present study the administration of *R.apiculata* extract significantly reduced the lung hydroxyproline level in metastatic tumor bearing animals. The *R.apiculata* extract inhibited excessive accumulation of collagen in lungs preventing pulmonary fibrosis during lung metastasis.

Hexosamine is the acidic and basic modification of monosaccharide yield uronic acids (glucoronic acid) and amino sugars (hexosamine). Hexosamine plays an important role in the production of N- acetyl neuraminic acid (sialic acid) which is present on the surface of malignant cells responsible for metastasis. Hexosamine, an integral part of many structural polysaccharides and glycosaminoglycans (GAG) found in the ground surface of ECM which promotes metastasis by opening the spaces for malignant cells to migrate. The excess hexosamine level is directly correlated with the active growth and proliferation of malignant cells (West et al., 1985). Therefore it is known marker and promoter of metastasis (Lipponen et al., 2001). In the study the administration of *R.apiculata* extract resulted in significant reduction in lung hexosamine level of metastatic tumor bearing animals. The *R.apiculata* extract significantly inhibited the excess synthesis of hexosamine by blocking the ECM thus preventing malignant cells metastasis and lung fibrosis (Pradeep and Kuttan, 2002).

Malignant cells yield uronic acid by the oxidation of the primary alcohol group of aldoses (monosaccharide) sugar derivatives. This leads to the formation of glucuronic acid lactone which is an essential form of uronic acid. In the present study the *R.apiculata* extract significantly decreased the lung uronic acid level in metastatic tumor bearing animals. The histopathological analysis of the lungs of *R.apiculata* treated animals also shows consonant results as above by reduction in the lung tumor nodules. Therefore, the above experimental evidences substantiate the anti-metastasis activity of the *R.apiculata* extract on experimentally induced lung metastasis in C57BL/6 mice.

In conclusion, this study is the first of its kind to report on the anti-metastatic activity of *R.apiculata* extract in metastatic tumor bearing animals. The inhibitory effect exhibited by *R.apiculata* extract could be attributed to the high content of pyrazole, 4-pyrrolidinyl, ketone derivatives and thiazolidinediones found in the methanolic extract (Prabhu and Guruvayoorappan, 2012). Therefore *R.apiculata* extract may be used as a therapeutic target to inhibit metastasis during tumor progression. Further investigations are required to trace out the exact mechanisms involved in the anti-metastatic property of *R.apiculata*.

Acknowledgements

The valuable guidance of Dr. V.M. Berlin Grace, (Head) Department of Biotechnology, and Dr. M. Patrick Gomez, Director, School of Biotechnology and Health Sciences, Karunya University is greatly acknowledged.

References

Ambs S, Merriam WG, Bennett WP, et al (1998). Frequent nitric oxide synthase-2 expression in human colon adenomas: implication for tumor angiogenesis and colon cancer progressionm. *Cancer Res*, **58**, 334-41.

Antony JJ, Sivalingam P, Siva D, et al (2011). Comparative evaluation of antibacterial activity of silver nanoparticles synthesized using *Rhizophora apiculata* and glucose. *Colloids Surf B Biointerfaces*, **88**, 134-40.

Aragon-Ching JB, Zujewski J (2007). CNS metastasis: an old problem in a new guise. *Clinical Cancer Res*, **13**, 1644-7.

Bergman I, Loxley R (1970). The determination of hydroxyproline in urine hydrosylates. *Clin. Chem Acta*, **27**, 347-9.

Bhatia S, Tykodll SS, Thompson JA (2009). Treatment of metastatic melanoma: an overview. *Oncology (Williston Park)*, **23**, 488-96.

Bhavanandhan VP, Katlic AW, Banks J, et al (1981). Partial characterization of sialoglycopeptides produced by cultured human melanoma cells and melanocytes. *Biochemistry*, **20**, 3586-94.

Bitter T, Muir HM (1962). A modified uronic acid carbazole reaction. Anal. *Biochem*, **4**, 330-34.

Branco-Price C, Zhang N, Schnelle M, et al (2012). Endothelial cell HIF-1α and HIF-2α differentially regulate metastatic success. *Cancer Cell*, **21**, 52-65.

Carretero J, Obrador E, Anasagasti MJ, et al (1999). Growth-associated changes in glutathione content correlate with liver metastatic activity of B16 melanoma cells. *Clin Exp Metastasis*, **17**, 567-74.

Chambers AF, Groom AC, MacDonald IC (2002). Dissemination

and growth of cancer cells in metastatic sites. *Nat Rev Cancer*, **2**, 563-72.

Cole SP (1986). Rapid chemosensitivity testing of human lung tumor cells using the MTT assay. *Cancer Chemother Pharmacol*, **17**, 259-63.

Edwards P, Cendan JC, Topping DB, et al (1933). A colourimetric method for determination of glucosamine. *J Biochem*, **27**, 1824-8.

Edwards P, Cendan JC, Topping DB, et al (1996). Tumor cell nitric oxide inhibits cell growth in vitro, but stimulates tumorigenesis and experimental lung metastasis in vivo. *J Surg Res*, **63**, 49-52.

Engbrin JA, Hoffman MP, Karmand AJ, Kleinman HK (2002). The B16F-10 receptor for a metastasis promoting site on Laminin-1 is a heparan sulfate/ chondroitin sulfate containing proteoglycan. *Cancer Res*, **62**, 3549-54.

Fukumura D, Kashiwagi S, Jain RK (2006). The role of nitric oxide in tumor progression. *Nat Rev Cancer*, **6**, 521-34.

Fuster MM, Esko JD (2005). The sweet and sour of cancer: Glycans as novel therapeutic targets. *Nature Reviews Cancer*, **5**, 526-42.

Green LC, Wagner DA, Glogowski J, et al (1982). Analysis of nitrate, nitrite and (15N) nitrate in biological fluids. *Anal Biochem*, **126**, 131-8.

Gupta GP, Massague J (2002). Cancer metastasis: building a framework. *Cell*, **127**, 679-95.

Herzig M, Christofori G (2002). Recent advances in cancer research: mouse models of tumorigenesis. *Biochim Biophys Acta*, **1602**, 97-113.

Hunter KW, Crawford NPS, Alsarraj J (2008). Mechanisms of metastasis. *Breast Cancer Res*, **10**, 2.

Hyoudou K, Nishikawa M, Umeyama Y, et al (2004). Inhibition of metastatic tumor growth in mouse lung by repeated administration of polyethylene glycol-conjugated catalase: quantitative analysis with firefly luciferase-expressing melanoma cells. *Clin Cancer Res*, **15**, 7685-91.

Khan N, Mukhtar H (2010). Cancer and metastasis: prevention and treatment by green tea. *Cancer Metastasis Rev*, **29**, 435-45.

Klein CA (2008). Cancer. The metastasis cascade. *Science* **321**,1785-87.

Kuttan R, Bhanumathy P, Nirmala K, George MC (1985). Possible anticancer activity of turmeric. *Nutr Cancer*, **29**, 197-202.

Lee JM, Yanagawa J, Peebles KA, et al (2008). Inflammation in lung carcinogenesis: new targets for lung cancer chemoprevention and treatment. *Crit Rev Oncol Hematol*, **66**, 208-17.

Leyon PV, Kuttan G (2004). Effect of Withania somnifera on B16F-10 meanoma induced metastasis in mice. *Phytother Res*, **18**, 118-22.

Leyon PV, Lini CC, Kuttan G (2005). Inhibitory effect of Boerhaavia diffusa on experimental metastasis by B16F10 melanoma in C57BL/6 mice. *Lifesciences*, **76**, 133-49.

Lipponen P, Aaltomaa S, Tammi R, Argen U, Kosma VM (2001). High stromal hyaluronan level is associated with poor differentiation and metastases in prostate cancer. *Eur J Cancer*, **37**, 849-56.

Luzzi KJ, MacDonald IC, Schmidt EE, et al (1998). Multistep nature of metastatic inefficiency: dormancy of solitary cells after successful extravasation and limited survival of early micrometastases. *Am J Pathol*, **153**, 865-73.

Mokkhasmit M, Swatdimongkol. K, Satrawaha P (1971). Study on toxicity of Thai medicinal plants. *Bull Dept Med Sci*. **12**, 36-65.

Nathan C (1992). Nitric oxide as a secretory product of mammalian cells. *FASEB J*, **6**, 3051-64.

Nelson DL, Cox MM (2005). Lehninger's Principles of Biochemistry, 4th Edition, W. H. Freeman and Company, New York.

Nguyen DX, Massagué J (2007). Genetic determinants of cancer metastasis. *Nat Rev Genet*, **8**, 341-52.

Nonaka Y, Iwagaki H, Kimura T, Fuchimoto S, Orita K (1993). Effect of reactive oxygen intermediates on the in vitro invasive capacity of tumor cells and liver metastasis in mice. *Int J Cancer*, **54**, 983-86.

Prabhu VV, Guruvayoorappan C (2010). Nitric oxide: Pros and cons in tumor progression. *Immunopharmacol Immunotoxicol*, **32**, 387-92.

Prabhu VV, Guruvayoorappan C (2012). Evaluation of immunostimulant activity and chemoprotective effect of mangrove *Rhizophora apiculata* against cyclophosphamide induced toxicity in BALB/c mice. *Immunopharmacol Immunotoxicol*, **34**, 607-14.

Prabhu VV, Guruvayoorappan C (2012a). Anti-inflammatory and anti-tumor activity of the marine mangrove *Rhizophora apiculata*. *J Immunotoxicology*, **9**, 341-352.

Prabhu VV, Siddikuzzaman, Grace VM, Guruvayoorappan C (2012b). Targeting tumor metastasis by regulating Nm23 gene expression. *Asian Pac J Cancer Prev*, **13**, 3539-48.

Pradeep K (2002). Effect of piperine on the inhibition of lung metastasis induced B16F-10 melanoma cells in mice. *Clinical & Experimental Metastasis*, **19**, 703-08.

Premanathan M, Arakaki R, Izumi H, et al (1999). Antiviral properties of a mangrove plant, *Rhizophora apiculata* Blume, against human immunodeficiency virus. *Antiviral Res*, **44**, 113-22.

Schantz SP, Brown BW, Lira E, Taylor SL, Beddingfield N (1987). Evidence for the role of natural immunity in the control of metastatic spread of head and neck cancer. *Cancer Immunology Immunotherapy*, **25**, 141-48.

Schauer R (1985). Sialic acids and their role as biological masks. *Trends Biochem Sci*, **10**, 357-60.

Shi Q, Huang S, Jiang W, et al (1999). Direct correlation between nitric oxide synthase II inducibility and metastatic ability of UV-2237 murine fibrosarcoma cells carrying mutant p53. *Cancer Res*, **59** , 2072-75.

Sithranga BN, Kandasamy K, Subramanian M, You-Jin J (2011). Effect of mangrove tea extract from ceriops decandra (Griff.) Ding Hou. on salivary bacterial flora of DMBA Induced hamster buccal pouch carcinoma. *Indian J Microbiol*, **51**, 338-44.

Skoza L, Mohos S (1976). Stable thiobarbituric acid chromophore with dimethyl sulphoxide. Application to sialic acid assay in analytical de o acetylation. *Biochem J*, **159**, 457-62.

Talmadge JE, Fidler IJ (2010). AACR centennial series: the biology of cancer metastasis: historical perspective. *Cancer Res*, **70**, 5649-69.

Tate SS, Meister A (1974). Inhibition of gamma glutamyl transpeptidase amino acids, peptides and derivatives and analogs of glutathione. *J Biochem*, **23**, 1602.

Thejass P, Kuttan, Girija (2006). Antimetastatic activity sulforaphane. *Life Sci*. **78**, 3043-50.

Vedolva E, Borovansky J (1994). Evaluation of serum sialic acid fractions as marker from malignant melanoma. *Cancer Lett*, **78**, 171-5.

Voet D, Voet JG (1995). Biochemistry, New York: John Wiley, 157-258.

Wang YP, Wang MZ, Luo YR, Shen Y, Wei ZX (2012). Lentivirus-mediated shRNA interference targeting SLUG inhibits lung cancer growth and metastasis. *Asian Pac J Cancer Prev*, **13**, 4947-51.

West DC, Hampson IN, Arnold F, et al (1985). Angiogenesis induced by degradation products of hyaluronic acid. *Science*,

228, 1324-26.
WHO glonocon report 2008.http://globocan.iarc.fr/factsheets/populations/factsheet.asp?uno=900
WHO Report (2008). National Policy on Traditional Medicine and Regulation of Herbal Medicines - Report of a WHO global survey.