ISSN: 0975-766X
CODEN: IJPTFI
Research Article

Available Online through
www.ijptonline.com

ANTIPROLIFERATIVE ACTIVITY OF METHANOLIC EXTRACTION OF INDIGOFERA TINCTORIA AGAINST B16F10 MELANOMA INDUCED LUNG METASTASIS IN C57BL/6 MICE

Dash S.L.[1]*, Pandey M.[1]*, Prusti A.K.[2], Samal H.B.[3]
[1]Maharana Pratap College of Pharmacy, Kanpur, Uttar Pradesh India.
[2]Aksharpreet Institute Of Pharmacy, Jamnagar, Gujarat, India.
[3]Guru Nanak Institute of Pharmacy, Ibrahimpatam, Andhar Pradesh, India.
Email: lanchhan4u@rediffmail.com

Received on 05-09-2011 *Accepted on 26-09-2011*

Abstract

The methanolic extraction of Indigofera tinctoria was studied for its antiprolifertive activity using B16F10 melanoma cells in C57BL/6 mice. Simultaneous administration of methanolic extraction at doses 100 and 200mg/kg, p.o significantly ($p < 0.01$) inhibited the metastatic colony formation of the melanoma in lungs by 47.54 and 69.52% respectively, with increase in the survival rate of the metastatic tumour bearing animals, as compared to the untreated control animals. Lung collagen hydroxyproline content was highly elevated in the control animals, which was reduced by the simultaneous administration of methanolic extraction at the tested dose levels. The level of lung hexosamines and uronic acid content was also elevated in the control animals. Administration of methanolic fraction of Indigofera tinctoria at tested dose levels 100 and 200 mg/kg, p.o significantly reduced the elevated level of hexosamine and uronic acid content, when compared to that of vehicle treated control animals. Levels of serum sialic acids and L-glutamyltranspeptidase that are markers of neoplastic proliferation were also reduced in the methanolic extraction treated animals as compared to the higher levels in the control animals. Histopathological analysis of the lung tissues also correlated with these findings. The *in vitro* cytotoxic activity of methanolic extraction of Indigofera tinctoria on B16F10 melanoma cells was studied using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue) assay and the IC50 was found to be 24.8 µg/ml. Thus,

simultaneous administration of methanolic fraction of Indigofera tinctoria at the tested dose levels was effective in inhibiting the metastasis of B16F10 melanoma cells and possessed significant antiproliferetive activity.

Key words: Indigofera tinctoria, B16F10 melanoma cells, metastasis, antiproliferetive.

Introduction

Indigo, after which this group of carbonyl dyes is named, is one of the oldest known natural dyes. It is a derivative of the colorless glucosides of the enol form of indoxyl. Indigo is formed from indican by fermentation of plant material such as *Indigofera tinctoria, Baphicacanthus cusia* Brem., *Indigofera suffruticosa, Polygonum tinctorium, Isatis indigotica.* Many studies have investigated the biosynthesis of indigo precursors and it was found that indole was precursor of indigo biosynthesis in plant Indirubin, which is a pinky-red pigment similar to indigo blue in structure was produced from *Polygonum tinctorium* Moreover, indirubin has been isolated from the crude extract of *Baphicacanthus cusia* Brem. Indirubin inhibited Lewis lung carcinoma in mice and Walker carcinosarcoma 256 in rats. In a clinical study, treatment with indirubin of patients with chronic myelocytic leukemia at doses of 300-450 mg daily showed 26% complete remissions and 33.4% partial remissions. In this study the indigo dye was obtained from fermentation of *Indigofera tinctoria* Linn. And *Baphicacanthus cusia* Brem. in methanol. The objective of this work is to develop the method of indigo extraction. Moreover, the separation and chemical structure analysis of dye components, comparision of the major components of the indigo dye obtained from *Indigofera tinctoria* Linn. and *Baphicacanthus cusia* Brem. and the preparation of the ready-to-use natural dye from these components are investigated.

Animals

Six to eight weeks old male C57BL/6 mice were purchased from CDRI, Lucknow, India. The animals were housed in well-ventilated cages kept in air-controlled rooms during the experiment. They were fed with normal mouse chow and water all the animal experiments were performed according to the rules and regulations of Animal Ethics Committee, Government of India.

Cells

B16F10, a highly metastatic melanoma cell line was purchased from CDRI, Lucknow, India. Cells were maintained in DMEM containing 2 mM L- glutamine, supplemented with 10% FBS, 100 units/ml penicillin and 100 g/ml streptomycin and incubated in a humidified atmosphere with 5% CO2 at 37°C.

Extraction

The plant material of Indigofera tinctoria leaf used for the investigation was collected from Costal region of Orissa. The plant was identified and authenticated by Prof. Dr. M. K. Misra, Botany Department of Berhampur University, Orissa and a voucher specimen is kept in the herbarium. The leaves were dried under shade and powdered in a mechanical grinder. The powdered material was extracted with methanol by soxhlet extraction. The methanol extract was concentrated using rotary evaporator and dried under vacuum. As the methanolic extract contains different type of phytochemicals, it was fractionated by hexane, pet-ether, chloroform, ethyl acetate and methanol. The various fractions were tested against a panel of cell lines representative of tumour from a variety of tissue types. Preliminary screening of the different solvent fractions of Indigofera tinctoria for cytotoxicity was studied against murine B16F10 melanoma cells and human cancer cell lines Lung (NCIH460), Colon (HCT116), Liver (HepG2) and Cervical (HeLa) using MTT assay method. The methanolic fraction of Indigofera tinctoria was found to be active with better cytotoxic activity in the panel of tested cancer cell lines when compared to that of other fractions of Indigofera tinctoria. Further preliminary phytochemical tests have been carried out for all the fractions (Data not shown in the manuscript). The methanolic fraction was found to be positive for the presence of Glycosides. So the methanolic fraction was used for further investigation.

MTT assay

Growth of B16F10 melanoma cells in the presence of Methanolic Extraction of Indigofera tinctoria was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue) assay. This assay was performed as described in the modified method of Mosmann (1983). Cells were seeded in 96 well microculture plate at 2 × 103 cells per well and allowed to adhere overnight. Cells were exposed to 0, 2.5, 5, 10, 50,

100 and 200 µg/ml of MFJC for 48 h. MTT was prepared at a concentration of 5 mg/ml in sterile phosphate buffered saline system (PBS). A 20 µl aliquot of the stock solution of MTT was added to each well. After 3 h of incubation at 37°C, 150 µl of DMSO was added to each well in order to dissolve the formazan crystals. Optical absorbancy were measured at 570 nm using a 96 well Spectramax Microplate Reader (Molecular Devices, Sunnyvale, CA, USA). The results obtained were calculated and presented as a percentage of control values.

***In vivo* study**

Highly metastatic B16F10 melanoma cells (5 × 105 cells/ 0.2 ml PBS) was injected to each animal via lateral tail vein on day 0 (Kobayashi et al., 1998). The animals were divided into four groups, comprising twelve animals in each group. An additional group of the same strain of animals (n = 6) were also kept without any treatment or tumour and used for the estimation of normal levels of different biochemical parameters. Group I was administered 2% v/v aqueous Tween 80, which served as control. In Group II and III MFJC at 100 and 200 mg /kg was administered orally to animals, simultaneously with the induction of metastasis and continued for 14 days. Group IV served as standard, which received Doxorubicin 4 mg/kg, i.p (Injections were on days 0, 4, 8 and 12 from tumor inoculation). Six animals from each group were sacrificed after 15 days of tumour induction, the lungs were dissected out and blood was collected. The number of blackish metastatic colonies present over the entire surface of the five lobes of the lung was counted under a dissecting microscope. Then the lung tissue was subjected to the estimation of collagen hydroxyproline (Bergman and Loxley, 1970), hexosamines (Elson and Morgan, 1933) and uronic acid (Bitter and Muir, 1962). The serum sialic acid levels of all the animals were determined according to the procedure of Skoza and Mohos (1976). The serum L-glutamyltranspeptidase (L-GT) (Szasz, 1976) levels were also assayed and expressed as units per litre.

Effect of methanolic fraction of Indigofera tinctoria **on the survival rate of metastatic tumour bearing animals**

Six animals of each group from the above experiment were observed for their survival rate. The mortality of each animals were observed and the percentage increase in life span (% ILS) was calculated from the formula:

$$\% \text{ ILS} = [(T- C/C)] \times 100$$

Where T represents the number of survival days of treated animals and C represent the number of survival days of control animals.

Histopathology of lungs

Lung tissues were fixed in 10% formaldehyde, dehydrated and embedded in paraffin wax. 4 μm sections were then stained with hematoxylin and eosin (H & E) mounted in DPX and examined under a microscope.

Statistical analysis

Statistical analysis results are presented as Mean ± S.D. Statistical evaluation of data was performed by using one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. $P < 0.05$ was considered statistically significant.

Results

Preliminary phytochemical test

Preliminary phytochemical screening of the methanolic fraction of the Indigofera tinctoria leaves revealed the presence of Glycosies.

Effect of MFJC on the lung tumour nodule formation

Earlier, it has been reported that B16F10 cells mainly form lung tumours (Engbrin et al., 2002). Administration of the methanolic extract of Indigofera tinctoria reduced pulmonary metastasis formation of B16F10 melanoma cells. Vehicle treated control animals developed massive number of tumour nodules on their lungs and the number of countable colonies were (264.67 ± 25.53). There was a significant ($P < 0.01$) reduction in the lung tumour nodule formation when the animals were simultaneously treated with MFJC at 100 and 200 mg/kg, p.o by 47.54 and 69.52% respectively in a dose dependent manner (Table 1). It was reduced to 30.66 ± 3.78 countable colonies by the doxorubicin treatment. The survival rate of the animals also increased to 43.14 and 66.25% by the simultaneous treatment with MFJC at 100 and 200 mg/kg, p.o respectively (Table 1). In doxorubicin treated group the survival rate of animals increased to 123.48%. In *in vitro* cytotoxic assay MFJC at tested concentrations (0 –

200 mg/ml) showed significant reduction in the proliferation of melanoma cells and the GI50 was found to be 24.8 μg/ml. Histopathology of the lung also showed marked reduction in tumour mass in the lungs of treated animals

Effect of MFJC on the lung collagen hydroxyproline content

Effect of MFJC treatment on the lung biochemical parameters is shown in Table 2. The lung collagen hydroxyl proline content was drastically elevated in the control group compared to that of normal level indicating the fibrosis of lung tissue. This elevated level was significantly ($P < 0.01$) reduced in the animals treated with MFJC 100 and 200 mg/kg, p.o in a dose dependent manner indicating the decreased metastatic lung fibrosis, comparable with that of doxorubicin.

Effect of MFJC on the lung uronic acid content

Tumour bearing control animals showed elevated levels of uronic acid in the lung tissue when compared with that of normal control (Table 2). Oral administration of MFJC at a dose of 100 and 200 mg/kg body weight significantly ($P < 0.01$) reduced these elevated levels in lung tissue. The results were found to be in a dose dependent manner, comparable with that of doxorubicin.

Effect of MFJC on the lung hexosamine content

There was an increased level of lung hexosamine content in the control tumour bearing animals, when compared with that of normal animals (Table 2). Treatment with MFJC at 100 and 200 mg/kg, p. o significantly ($P < 0.01$) of normal control (Table 2). Oral administration of MFJC at a dose of 100 and 200 mg/kg body weight significantly ($P < 0.01$) reduced these elevated levels in lung tissue. The results were found to be in a dose dependent manner, comparable with that of doxorubicin.

Effect of MFJC on the serum sialic acid level

The serum sialic acid level was drastically elevated in the control group when compared to that of normal animals. MFJC (100 and 200 mg/kg, p.o) treated animals showed a significant ($P < 0.01$) reduction in the sialic acid content. The elevated sialic acid levels in the metastatic tumour bearing animals were significantly ($p < 0.01$) reduced to near normal upon treatment with doxorubicin

Effect of MFJC on serum GGT level

The GT activity in the control animals was very high as compared to the γ-GT level in the serum of the normal animals. The higher content of γ- GT was significantly ($P < 0.01$) reduced to near normal, when the animals were simultaneously treated with MFJC at tested dose levels and the results were comparable with that of doxorubicin.

Histopathological analysis of lung

The hematoxylin and eosin stained sections of lung tissues are shown in. The lung from healthy normal animal shows normal architecture of lungs with bronchioles, alveoli and interstitium . The lungs of vehicle control animals showed massive tumour growth around the bronchioles and infiltration of metastatic colonies of melanoma in the interstitium of the lung. Increased fibrosis reduces alveolar space, which leads to reduction in vital capacity of the lung. Simultaneous administration of MFJC at 100 and 200 mg/kg, p.o showed significant reduction in tumor mass and regeneration of alveolar passage with ciliated columnar epithelial cells. Lungs of the Doxoubicin treated animals were almost similar to the healthy normal lung

MFJC- methanolic extraction of Indigofera tinctoria

B16F10 cells were treated with different concentrations of MFJC (0 – 200 _g/ml) for 48 h. Control cells were maintained in the medium for the indicated time period. Results are expressed as a percentage of vehicle-treated control and Mean ± S.D of three separate experiments. The lungs were dissected out and observed for metastasis on 15th day after induction of B16F10 melanoma (5 × 105 cells). MFJC treatment (100 and 200 mg/kg) started simultaneously with tumour cell induction through the oral administration (14 doses at 24 h interval, p.o). bIncrease in life span = (T-C/C) × 100, where T and C are the number of days survived by the treated and control (vehicle treated) group of animals respectively. *$P < 0.01$ statistically significant when MFJC treated groups and doxorubicin groups are compared with vehicle control.

Table-1: Effect of methanolic extract of Indigofera tinctoria on lung colonization of B16F10 melanoma cells and survival of the animals.

Treatment	No. of metastatic colonies	% Inhibition	No. of days survived	%ILS$_b$
Vehicle Control	264.67 ± 25.53	-	36.92 ± 5.1	-
MFJC 100 mg/kg, p.o	138.83 ± 8.66*	47.54	52.85 ± 6.2*	43.14
MFJC 200 mg/kg, p.o	80.67 ± 7.74*	69.52	61.38 ± 7.7*	66.25
Doxorubicin 4 mg/kg,i.p	30.66 ± 3.78*	88.41	82.51 ± 8.6*	123.48

Values are expressed as Mean ± SD. MFJC- methanolic fraction of Indigofera tinctoria. a The lungs were dissected out and observed for metastasis on 15th day after induction of B16F10 melanoma (5 × 105 cells). MFJC treatment (100 and 200 mg/kg) started simultaneously with tumour cell induction through the oral administration (14 doses at 24 h interval, p.o). bIncrease in life span = (T-C/C) × 100, where T and C are the number of days survived by the treated and control (vehicle treated) group of animals respectively. *P < 0.01 statistically significant when MFJC treated groups and doxorubicin groups are compared with vehicle control. Values are expressed as Mean ± SD.

Table-2: Effect of methanolic fraction of Indigofera tinctoria administration on the lung biochemical parameters of metastasis bearing animals.

Treatment	Lung collagen hydroxy praline (µg/mg protein)	Lung uronic acid (_g/100 mg)	Hexosamines (mg/100 mg)
Normal	1.84 ± 0.42	36.41 ± 3.6	0.61 ± 0.1
Vehicle Control	18.27 ± 1.38	244.92 ± 15.1	5.14 ± 0.68
MFJC 100 mg/kg, p.o	10.16 ± 0.98*	152.39 ± 7.4*	2.46 ± 0.18*
MFJC 200 mg/kg, p.o	7.18 ± 0.82*	108.13 ± 9.1*	1.53 ± 0.16*
Doxorubicin 4 mg/kg, i.p	4.93 ± 0.28*	71.24 ± 6.8*	1.18 ± 0.05*

Values are expressed as Mean ± SD. MFJC- methanolic fraction of *Indigofera tinctoria*. *P < 0.01 statistically significant when MFJC treated groups and doxorubicin groups are compared with vehicle control.

Discussion and Conclusion

B16F10 melanoma cells are highly metastatic and form colonies of tumour nodules in the lungs when administered through tail vein, which in turn promote lung fibrosis and collagen deposition. In the arliest postinjection time points, the majority of cells find themselves in the pulmonary tissue, but some are also localized in other organs. After 14 days, only the lungs contain B16 cells, now seen as tumour nodules and none of the other

tissues show the establishment of tumours (Fidler, 1970). In the present study, we analyzed the anti-metastatic activity of methanolic fraction of *Indigofera tinctoria* in B16F10 melanoma induced lung metastasis. Oral administration of MFJC at 100 and 200 mg/kg, p.o resulted in marked reduction in the metastasis by B16F10 melanoma. Exuberant collagen deposition within the lung is a key marker of fibrosis (Phan, 2003). The lung fibrosis was evaluated by estimating the lung collagen hydroxyproline content, because during lung fibrosis collagen is deposited massively in the alveolus of lungs. Fifteen to thirty percent of collagen is hydroxyproline (Voet and Voet, 1995) and accumulation of extracellular matrix, especially collagen, will reduce the pulmonary function. There was an elevated level of lung collagen hydroxyproline content in metastasis bearing animals. Treatment with MFJC at 100 and 200 mg/kg, p.o, caused significant reduction in the accumulation of collagen, as estimated from the hydroxyproline content. These findings correlate with the reduction in the number of lung tumour nodules in the treated groups. Histopathological analysis of lungs correlates with these findings. Hyaluronan (HA) is a nonsulfated, linear glycosaminoglycan, consisting of repeating units of (-,1-4)-D-glucuronic acid(-,1-3)-N-acetyl-D-glucosamine (Brinck and Heldin, 1999) and is one of the chief components of the extracellular matrix, contributes significantly to cell proliferation and migration and may also be involved in the progression of some malignant tumours (Stern, 2004). The acidic and basic modifications of nosaccharides 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. Tumour cells can induce the host stromal cells to supplement the matrix components necessary for the growing tumour (McKinnell et al., 1998). Concentration of HA is elevated in several cancers regardless of the tumour grade (Hautmann et al., 2000; Setala et al., 1999). The degradation products of hyaluronic acid (HA), oligoHA, are also known to stimulate endothelial-cell proliferation and to promote neovascularization associated with angiogenesis which in turn promotes tumour metastasis and tumour directed angiogenesis (West et al., 1985; Slevin et al., 2002).

There was an elevated level of these monosaccharides in the control metastatic tumour bearing animals which clearly indicates the active growth and proliferation of tumour cells. Administration of methanolic fraction of

Indigofera tinctoria significantly reduced the uronic acid and hexosamine content in the tumour bearing animals. The inhibitory effect of methanolic fraction of *Indigofera tinctoria* on proliferation of B16F10 melanoma cells (*in vitro*) strongly supports these results. Hexosamine has an important role in the synthesis of sialic acid, a family of acetylated derivatives of N-acetyl neuraminic acid, occurs as a terminal component of carbohydrate chain of glycolproteins and gangliosides. It forms structural component of glycolipids present on the surface of tumour cells (Voet and Voet, 1995; Erbil et al., 1985). Various studies have reported the significance of sialic acid as a tumour marker in various cancers including melanoma. Aberrant glycosylation processes in tumour cells contribute to the biosynthesis of certain oligosaccharides; hence, malignant or transformed cells contain increased sialic acid residues on their surfaces (Yogeeswaran, 1981; Hulbert et al., 1979). Sialic acid levels also served to monitor treatment of cancer. Cancer cells that can metastasize often have a lot of sialic acid-rich glycoproteins. This helps these latestage cancer cells enter the blood stream (Baxi et al., 1990; Tewarson et al., 1993; Patel et al., 1997; Bathi et al., 2001). In the present study, sialic acid level was drastically increased in the control metastatic tumour bearing animals when compared to that of normal animal. The elevated sialic acid levels were ignificantly reduced in the animals treated with methanolic fraction of *Indigofera tinctoria*. Serum g-glutamyltranspeptidase (GGT) is also a marker of neoplastic proliferation. A close association between the metastatic ability of B16 melanoma sub lines and expression of membrane-associated GGT has been reported previously (Prezioso et al., 1993). GGT catalyses intracellular GSH break down and provide energy to the tumour cells by L-glutamyl cycle (West et al., 1985). High level of GGT was found in the serum of control metastatic tumour bearing animals when compared with that of normal animals. Administration of methanolic fraction of *Indigofera tinctoria* at 100 and 200 mg/kg, p.o significantly reduced the serum GGT level. All these results correlated with the inhibition of lung tumour nodules and the increase in life span of metastatic tumour bearing animals, when treated with methanolic fraction of *Indigofera tinctoria*. Histopathological analysis also supports these findings. Cancer metastasis, which involves multiple processes and various cytophysiological changes, is a primary cause of cancer death. Currently available therapeutic drugs have limited effects on metasatic tumours. Therefore, there is an urgent need for novel therapeutic

approaches to treat tumour metastasis. Search for effective agent from plant resources, such as flavonoids and another phytochemicals, for treatment of cancer metastasis has become one of the top priorities in cancer research. Flavonoids, a family of phytochemical compounds, are widely distributed in foods of plant origin such as vegetables, fruits and in medicinal plants, display a wide range of pharmacological properties including antioxidative, anti-inflammatory, antiproliferative, antimutagenic, anti-carcinogenic and anti-cancer effects (Kuo, 1997; Birt et al., 2001; Ren et al., 2003; Yamamoto and Gaynor, 2001; Li et al., 2007). Accumulating evidence from *in vitro* and animal studies suggest that some plant flavonoids possess potent cancer chemoprevention activities, anti-invasive activities *in vitro* and antimetastasis activities in animal models (Taniguchi et al., 1992; Caltagirone et al., 2000; Iwashita et al., 2000). Recently more research has been focused on the role of flavonoids in cancer prevention because epidemiological investigations suggest that increased intake of fruits and vegetables are associated with the reduced risks of certain cancers. In preliminary phytochemical studies the methanolic fraction of *J. curcas* used in this study showed positive results for the presence of flavonoids, which correlates with earlier reports. Thus, the antimetastatic activity may be in part due to the presence of these flavonoids. Further studies have to be conducted to find out the exact mechanism of action of the phyto phytochemical(s) in inhibiting the cascade of event of metastasis.

References

1. Bathi RJ, Nandimath K, Kannan N, Shetty P (2001). Evaluation of glycoproteins as prognosticators in head and neck malignancy. Indian. J. Dent. Res. 12: 93-99.
2. Baxi BR, Patel PS, Adhavaryu SG (1990). A report on clinical importance of serum glycoconjugates in oral cancer. Indian. J. Clin.Biochem. 5: 139-144.
3. Beyioku AF, Oyibo WA, Anuforom BC (1998). Disinfectant/antiparasitic activities of *Jatropha curcas*. East. Afr. Med. J. 75: 508-511.
4. Birt DF, Hendrich S, Wang W (2001). Dietary agents in cancer prevention: flavonoids and isoflavonoids. Pharmacol. Ther. 90(2-3): 157-177.

5. Brinck J, Heldin P (1999). Expression of recombinant hyaluronan synthase (HAS) isoforms in CHO cells reduces cell migration and cell surface CD44. Exp. Cell. Res. 252(2): 342-351.
6. Caltagirone S, Rossi C, Poggi A, Ranelletti FO, Natali PG, Brunetti M, Aiello FB, Piantelli M (2000). Flavonoids Apigenin and Quercetin Inhibit Melanoma Growth and Metastatic Potential. Int. J. Cancer. 87(4): 595-600.
7. Cavallaro U, Christofori G (2001). Cell adhesion in tumour invasion and metastasis: loss of the glue is not enough. Biochim. Biophys. Acta. 1552: 39-45.
8. Chhabra SC, Mahunnah RLA, Mshiu EN (1990). Plants used in traditional medicine in Eastern Tanzania. III. Angiosperms (Euphorbiaceae to Menispermaceae). J. Ethnopharmacol. 28(3): 255-83.
9. Diepgen TL, Mahler V (2002). The epidemiology of skin cancer. Br. J. Dermatol. 146(61): 1-6.
10. Duke JA (2001). Handbook of Medicinal Herbs. CRC Press, London. pp. 253-5.
11. Engbrin JA, Hoffman M.P, Karmand AJ, Kleinman HK (2002). The B16F10 cell receptor for a metastasis promoting site on Laminin-1 is a heparan sulfate / chondroitin sulfate containing proteoglycan. Cancer Res. 62: 3549–3554.
12. Fidler IJ (1970). Metastasis: quantitative analysis of distribution and fate of tumour emboli labeled with 125I-5-iodo-2_-deoxyuridine. J. Natl.Cancer. Inst. 45: 773-782.
13. Garbe C, McLeod GR, Buettner PG (2000). Time trends of cutaneous melanoma in Queensland, Australia and Central Europe. Cancer. 89: 1269-1278.
14. Grosvenor PW, Supriono A, Gray DO (1995). Medicinal Plants from Riau province, Sumatra, Indonesia. Part II: antibacterial and antifungal activity. J. Ethnopharmacol. 45: 97-111.
15. Gupta MP, Monge A, Karikas GA, Cerain A, Solis PN, Leon E, Trujillo M, Suarez O, Wilson F, Montenegro G, Noriega Y, Santana AI, Correa M, Sanchez C (1996). Screening of Panamanian medicinal plants for brine shrimp toxicity, crown gall tumour inhibition, cytotoxicity and DNA intercalation. Int. J. Pharmacog. 34: 19-27.

16. Ha KT, Kim JK, Lee YC, Kim CH (2004). Inhibitory effect of Daesungki-Tang on the invasiveness potential of hepatocellular carcinoma through inhibition of matrix metalloproteinase-2 and -9 activities. Toxicol. Appl. Pharmacol. 200: 1-6.
17. Hautmann SH, Lokeshwar VB, Schroeder GL, Civantos F, Duncan RC. Friedrich MG, Soloway MS (2001). Elevated tissue expression of hyaluronic acid and hyaluronidase validate HA-HAase urine test for bladder cancer. J. Urol. 165: 2068-2074.
18. Iwashita K, Kobori M, Yamaki K, Tsushida T (2000).Flavonoids inhibit cell growth and induce apoptosis in B16 melanoma 4A5 cells. Biosci. Biotechnol. Biochem. 64(9): 1813-1820.
19. Kambu K, Tona L, Kaba S, Cimanga K, Mukala N (1990). Antispasmodic activity of extracts proceeding of plant anti-diarrhea traditional preparations used in Kinshasa, Zaire. Ann. Pharm. Fr. 48:200-208.
20. Khan M, Jain DC, Bhauni RS, Zaim M, Thakur RS (1991). Occurrence of some antiviral sterols in *Artemisa annua*. Plant. Sci. 75: 161-165.
21. Kobayashi M, Kobayashi H, Pollard RB, Suzuki F (1998). A pathologic role of Th2 cells and their cytokine products on the pulmonary metastasis of murine B16 melanoma. J. Immunol. 160(12): 5869-5873.
22. Kuo SM (1997). Dietary flavonoid and cancer prevention: evidence and potential mechanism. Crit. Rev. Oncog. 8(1): 47-69
23. Lens MB, Dawes M (2004). Global Perspectives of Contemporary Epidemiological Trends of Cutaneous Malignant Melanoma. Br. J. Dermatol. 150(2): 179-185.
24. Li Y, Fang H, Xu W (2007). Recent advance in the research of flavonoids as anticancer agents. Mini. Rev. Med. Chem. 7(7): 663- 678.
25. Liotta LA, Kohn EC (2003). Invasion and metastasis. In: Kufe DW, Pollock RE, Weichselbaum RR, Bast Jr RC, Gansler TS, Holland JF, Frei III E (eds) Holland Frei Cancer Medicine 6. BC Decker Inc.Hamilton, Canada. Chapter 122.
26. Mays SR, Nelson BR (1999). Current therapy of cutaneous melanoma. Cutis. 63: 293-298.

27. McKinnell RG, Parchment RE, Perantoni AO, BurryPierce G (1998). The biological basis of cancer. Cambridge University Press, UK. Morton DL, Essner RE, Kirkwood JM, Wollman RC (2003). Malignant melanoma. In: Kufe DW, Pollock RE, Weichselbaum RR, Bast Jr RC, Gansler TS, Holland JF, Frei III E (eds) Holland Frei Cancer Medicine 6. BC Decker Inc: Hamilton, Canada, Chapter 10.
28. Patel PS, Raval GN, Patel MM, Balar DB, Patel DD (1997).Importance of glycoproteins in human cancer. Indian. J. Biochem. Biophys. 34: 226-233.
29. Phan SH (2003). Fibroblast phenotypes in pulmonary fibrosis. Am. J. Respir. Cell Mol. Biol. 29 (3): S87-S92.
30. Poste G, Fidler IJ (1980). The pathogenesis of cancer metastasis. Nature. London. 283: 139-146.
31. Prezioso JA, Wang N, Duty L, Bloomer WD, Gorelik E (1993). Enhancement of pulmonary metastasis formation and gammaglutamyltranspeptidase activity in B16 melanoma induced by differentiation *invitro*. Clin. Exp. Metastasis. 11: 263-274.

Corresponding Author:
Dash S.L.*,
Email: *lanchhan4u@rediffmail.com*