Cytotoxic Pyrrolidinone from Leaves of *Jatropha tanjorensis*

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**Abstract**—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 metastasis tumors. Therefore, there is an urgent need for novel therapeutic approaches to treat tumor metastasis. Search for effective agent from plant resources, such as flavonoids or alkaloids for the treatment of cancer metastasis has become one of the top priorities in cancer research. The aim of the present study is to study the cytotoxic effects of leaves on *Jatropha tanjorensis* by *in vitro* and *in vivo* methods. *In vitro* cytotoxic properties of methanol extract and R (+) 4-hydroxy-2-pyrrolidinone of *J. tanjorensis* were taken up against 4 cancerous cell lines, viz., HEP-2, B16F10, A549, and NRK 49F using standard procedure. According to Hartwell latent sap and leaf of species of *Jatropha* are used in warts and tumors. This plant has not been studied for their cytotoxic effect. The methanol fraction of *J. tanjorensis* was studied for *in vivo* cytotoxic activity using B16F10 melanoma cells in mice. Simultaneous administration of methanol fraction at the doses of 100 and 200 mg/kg and R (+) 4-hydroxy-2-pyrrolidinone per oral. The methanol extract and R (+) 4-hydroxy-2-pyrrolidinone exhibited more cytotoxic activity against all the 4 cell lines by *in vitro* study. The Caucasian male larynx epithelium cornicing Hep-2 was found to be more susceptible with an IC₅₀ value of 43.24 μg/mL. The other cell line showed less activity as indicated by the relatively high IC₅₀ value. The results *in vivo* study revealed that R (+) 4-hydroxy-2-pyrrolidinone exhibited relatively high increase in life span than methanol fraction.

Key words: Jatropha tanjorensis, R (+) 4-hydroxy-2-pyrrolidinone in- vitro & in- vivo cytotoxic activity

I. INTRODUCTION

The genus *Jatropha* belongs to tribe Joanneasiae of Crotonoideae in the Euphorbiaceae family and contains approximately 175 species, cultivated throughout the tropical to temperate regions of the world. The name is derived from the Greek words ιατρός (iatros), meaning "physician," and τροφή (trophe), meaning "nutrition". *Jatropha tanjorensis* J.L. Ellis & Saroja in J. Bombay Nat. Hist. Soc. 58: 834. 1962. Shrubs, 3-4 m high; stem long, stout, dichotomously branched; branches puberulous when young, glabrous when mature. Distribution: INDIA: Tiruchirappalli, Pudukottai, Thanjavur and Ramanathapuram Districts in Tamil Nadu and Pondicherry. West Africa including Nigeria: Weed of field crops, bush re-growth, roadside and disturbed places in the higher rainfall zones. In Southwest Nigeria, infusion of *J. tanjorensis* leaves is taken orally for the treatment of diabetic symptoms. Olayiwola et al. (2004) assessed the three fractions of the leaf extract for their anti-diabetic potentials by in-vitro models. Viswanathan and Jeya Ananthi (2009) evaluated the activity of the hexane, chloroform and methanol leaf extracts against Staphylococcus aureus, Bacillus subtilis, Escherichia coli and Pseudomonas aeruginosa and their anti-inflammatory activity against carrageenin-induced hind paw edema in rats. All the extracts exhibited varying degree of antibacterial activity and anti inflammatory activity. Idu et al. (2009) investigated the morphological and anatomical studies of the leaves and stem of *J. curcas* and *J. tanjorensis*. Oboh and Masoadej (2009) reported activity of the aqueous leaf extract against *Staphylococcus aureus* and *Escherichia coli* as 1.6 and 1.3 cm respectively. Omobuwajo et al. (2011) investigated the acute, sub-acute toxicity and microscopy of the leaves in *J. tanjorensis*. Viswanathan and Jeya Ananthi (2012) evaluated Antimicrobial activity of bioactive compounds and leaf extracts in *Jatropha tanjorensis*.

administration of methanol fraction at the doses of 100 and 200 mg/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. A 10% aqueous infusion increases cardiac contraction in small doses (Horacio et al., 1974), and it is used against warts and cancers, Ethanol extract of defatted leaves and twigs active in vivo and in vitro against P-388 lymphocytic leukemia; see also tumor-promoting antileukemic diterpenoids (Hartwell, 1969; Hufford and Oguntimenn, 1978) Seed is used against warts and cancers (Hartwell, 1969). Seed is used against warts and cancers (Hartwell, 1969). Sap is used in cancer (Hartwell, 1969). Pharmacological mechanisms of an alkaloid, tetramethylpyrazine, have been reported in hypotensive, neuromuscular and cardiovascular actions by Ojewole and Odebiyi (1980, 1981). Ethanol extract of the aerial parts (5%) is treated for CNS depression and diuretic (Dhawan et al., 1977).

The aim of the present study is to study the cytotoxic effects of leaves on Jatropha tanjorensis taken up against 4 cancerous cell lines, viz., HEP-2, B16F10, A549, and NRK 49F using standard procedure. This plant has not been studied for their cytotoxic effect.

II. MATERIALS AND METHODS

2.1 Plant Material:

Leaves of Jatropha tanjorensis were collected in the vicinity of Pondicherry during August and September 2004. An authentic herbarium specimen (MBV & JJ 14774) was prepared and deposited in the Herbarium of the Centre for Research and Development in Siddha-Ayurveda Medicines (CRDSAM), Department of Plant Science, Bharathidasan University, Tiruchirappalli 620 024, Tamil Nadu, India, for reference.

2.2 Preparation of Plant Extracts:

The collected plants were dried at room temperature, pulverized by a mechanical grinder, sieved through 40 mesh. The powdered materials were extracted with hexane, chloroform and methanol using Soxhlet extraction apparatus. The chloroform and methanolic extract was then concentrated and dried under reduced pressure. The methanol free semisolid mass thus obtained was used for the experiment (Harborne, 1998).

2.3 Pharmacology:

Toxicity of both the extracts and isolated compounds was fixed for biological evaluation following the Organization for Economic Cooperation and Development (OECD) guidelines (2001). LD_{50} values were calculated with no sign of acute toxicity at >2000 mg/kg for the extracts and 10 mg/kg for the isolated compounds. Animal studies were performed with the approval of the Institutional Animal Ethics Committee (IAEC) in Arulmigu Kalasalingam College of Pharmacy, Krishnan Koil 626126 (Reg. No. 509/01/C/CPCSEA - Committee for the Purpose of Control and Supervision on Experimental Animals, Department of Animal Welfare, Government of India (No.412). Experimental animals housed under standard conditions were fed with standard diet (Lipton India Ltd., Bangalore) and water ad libitum in the Animal House, and maintained at room temperature under suitable nutritional and environmental conditions throughout the experiment. Fine chemicals were purchased from Sigma-Aldrich, St. Louis, MO 63103, and S.D. Fine Chemicals, Mumbai, India, and other chemicals from SISCO Research Laboratories Pvt. Ltd., Mumbai, India.

2.4 In Vitro Cytotoxic Activity:

In-vitro cytotoxic properties of methanol extract and R(+)-4-hydroxy-2-pyrolidinone of J. tanjorensis were taken up against 4 cancerous cell lines, viz., HEP-2, B16F10, A549, and NRK 49F using standard procedure. Each extract was separately dissolved with distilled dimethyl sulphoxide (DMSO) and the volume was made up to 10 ml with Dulbecco’s modified Eagle’s medium (DMEM, pH 7.4, supplemented with 2% motivated newborn calf serum, to obtain a stock solution of 1 mg/ml concentration sterilized by filtration and stored at -2°C till use. The cell cultures were propagated in DMEM, pH 7.4 supplemented with 10% inactivated newborn calf serum, penicillin (100 iu/ml), streptomycin (100 µg/ml and amphotericin B (5 µg/ml) and maintained at a humidified atmosphere of 5% CO₂ at 37°C until confluent. The cells were dissociated with 0.2% trypsin and 0.02% EDTA in phosphate buffer saline solution. The stock culture was grown in 25 cm² tissue culture flasks and all cytotoxicity experiments were carried out in 96 well microtitre plates. DLA cells used were propagated and maintained in the peritoneal cavity of Swiss albino mice. Cell lines in exponential growth phase were washed, trypsinized and resuspended on DMEM medium with 10% inactivated newborn calf serum. Cells were placed on 10,000 cells/well in 96 well microtitre plate and incubated for 24 h at 37°C. A 5% CO₂ in humidified atmosphere during which period a partial monolayer was formed. The cells were then exposed to different concentrations (1000 µg/ml to 15.6 µg/ml, prepared by serial two fold dilution using maintenance medium from the stock solution) of the test extracts on quadruplicate. Control received only maintenance medium. The cells were incubated at 31°C in the humidified incubator with 5% CO₂ for a period of 72 h. Morphological changes of the cell cultures were examined using an inverted tissue culture microscope at 24 h time intervals and compared with control. At the end of 72 h, cellular viability was determined using standard 3-(4, 5, dimethylthiazol-2-Yl-2 phenyl tetrazolium bromide (MTT) and sulphorhodamine B (SRB) assays. The short term toxicity studies were carried out by employing DLA cells. Trypan blue dye exclusion technique and the CTC-50 value (concentration of the sample required to kill 50% of the cells) were calculated.
2.5 In-Vivo Cytotoxic Activity:

The methanol fraction of *J. tanjorensis* was studied for cytotoxic activity using B16F10 melanoma cells in mice. Simultaneous administration of methanol fraction at the doses of 100 and 200 mg/kg and R(+) 4-hydroxy-2-pyrrolidinone per oral.

2.6 Cells:

B16F10, a highly metastatic melanoma cell line was purchased from National Centre for Cell Sciences, Pune, India. Cells were maintained in DMEM containing 2 mM – L glutamine, supplemented with 10% FBS (Fetal bovine serum), 100 units/ml penicillin and 100 μg/ml streptomycin and incubated in a humidified atmosphere with 5% CO2 at 37°C.

2.7 In Vivo Study:

Highly metastatic B16F10 Melanoma cells (5X10^5 cells/0.2 ml PBs) were injected into each animal via lateral tail vein on day 0 (Shi et al., 1998).

The animals were divided into 5 groups comprising 6 animals in each group. Group 1 was administered 2% v/v aqueous Tween80, which served as Control. In groups 2 and 3, methanol extract at 100 and 200 mg/kg was administered orally to animals and group 4 was given with R(+) 4-hydroxy-2-pyrrolidinone. Group 5 served as Standard, which received doxorubicin 4mg/kg i.p. (on days 0, 4, 8 and 12 from tumour inoculation), with the induction of metastasis and continued for 14 days. The animals of each group were observed for their survival rate. The mortality of each animal was observed and the percentage increase in life span (% ILS) was calculated from the formula %/ILS = [(T-C/C)] X 100; T - represents the number of survival days of treated animals; and C - represents the number of survival days of Control animals.

III. RESULTS

3.1 Cytotoxic Activity:

3.1.1 In-vitro Cytotoxic Activity:

The IC50 ± S.E.M. (By MTT 3-[4, 5, dimethyl/thiazol-2-yl]-2-phenyl tetrazolium bromide assay) of methanol extract in cell line Hep-2 was 43.24 ± 0.01. IC50 ± S.E.M. (by SRB sulforhodamine B assay) of methanol extract in cell line Hep-2 was 40.24 ± 0.02. The assay value in cell line B16F10 was 49.60 ± 0.03 by MTT assay and 43.54 ± 0.04 by SRB assay. IC50 ± S.E.M. values in cell line A549 were 58.85 ± 0.01 by MTT assay and 47.76 ± 0.06 by SRB assay. IC50 ± S.E.M. values in cell line KR were 53.65 ± 0.02 by MTT assay and 44.46 ± 0.04 by SRB assay. IC50 ± S.E.M. values of R(+) 4-hydroxy-2-pyrrolidinone in cell line Hep-2 were 42.26 ± 0.03 by MTT assay and 40.04 ± 0.01 by SRB assay. IC50 ± S.E.M. values in cell line B16F10 were 44.56 ± 0.02 by MTT assay and 42.32 ± 0.02 by SRB assay. IC50 ± S.E.M. values in A549 were 48.26 ± 0.03 by MTT assay and 45.46 ± 0.04 by SRB assay. IC50 ± S.E.M. values in cell line KR were 47.28 ± 0.03 by MTT assay and 42.26±0.04 by SRB assay.

The methanol extract and R(+) 4-hydroxy-2-pyrrolidinone exhibited more cytotoxic activity against all the 4 cell lines. The Caucasian male larynx epithelium cornicing Hep-2 was found to be more susceptible with an IC50 value of 43.24μg/ml. The other cell line showed less activity as indicated by the relatively high IC50 value (Tables 1, 2; Figs. 1, 2).

3.1.2 In-vivo Cytotoxic Activity

The effect of methanol fractions on lung melanoma of B16F10 and survival of animals (in days) was 53.46 ± 3.4 (42.59 % ILS) at 100 mg/kg and 60.24 ± 4.2 (61.08 % ILS) at 200 mg/kg. R(+) 4-hydroxy-2-pyrrolidinone at 10 mg/kg produced 62.12 ± 7.6 (66.00% ILS) and standard drug doxorubicin (4 mg/kg) showed an effect of 83.41 ± 4.2 (122.90% ILS). The results of the present study revealed that R(+) 4-hydroxy-2-pyrrolidinone exhibited relatively high increase in life span than methanol fraction (Table 3; Fig. 3).

IV. DISCUSSION

4.1 Cytotoxic Activity:

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 metastasis tumors. Therefore, there is an urgent need for novel therapeutic approaches to treat tumor metastasis. Search for effective agent from plant resources, such as flavonoids or alkaloids for the treatment of cancer metastasis has become one of the top priorities in cancer research.

The use of latex on warts, whitlows and the like is worldwide (Hartwell, 1969). Number of melanoma cases is increasing faster than any other cancer worldwide (Diepgen and Mahler, 2002). The estimates suggested doubling of melanoma incidence every 10-20 years (Garbe et al., 2000). Despite improved survival rates, the death rate from cutaneous metastatic melanoma (CMM) continues to climb as a result of exponential increases in incidence, making it a major public health problem for the foreseeable future (Lens and Dawes, 2004). Melanoma now accounts for approximately 4% of all cancers diagnosed in the United States. When diagnosed early in the course of the disease, melanoma is readily cured by simple wide surgical excision. However, once melanoma metastasizes, no currently available treatment reliably affects the course of disease. At the time of autopsy, the lung, liver, brain and lymph nodes are the most common sites of metastasis. Patients with pulmonary metastasis have a median survival period of 8 – 10 months (Morton et al., 2003). Any agent or drug which can interfere with any of these steps can significantly reduce the metastatic potential and can be useful in the inhibition of tumor metastasis. Several attempts have been made to inhibit tumor metastasis preventing the formation of tumors and tumor invasion using herbs (Yang et al., 2003; Ha et al., 2004). Anti-metastatic potential of curcasone B, a diterpene of *J. curcas* was investigated in...
many tropical countries against 4 human cancer cell lines such as KB, MCF-7, KFU-100 and MEM and reported a strong reduction of in vitro invasion, motility and secretion of matrix-metalloproteinases (MMP) of the cancer cells and non-toxicity to cells (Surin et al., 2005).

Compounds isolated from J. curcas leaves included flavonoids — apigenin and its glycosides, vitexin and isovitexin and sterols — stigmasterol, β - D - sitosterol and its β - D - glycoside (Subramanian et al., 1971 b; Khafagy et al., 1977; Chhabra et al., 1990; Saxena et al., 2005). Further, anti-crustacean activity, DNA intercalating effect and anti-viral activity are reported (Khan et al., 1991; Gupta et al., 1996). Its butanolic leaf extract and decoction of dried root bark were reported to have antispasmodic activity (Kambu et al., 1990). Its methanol extract of defatted leaves and twigs showed antileukemic activity (Hufford and Oguntimein, 1978). Its leaf extract was confirmed to possess disinfectant/antiparasitic activity (Beyioku et al., 1998). Its extracts of dried leaves, dried latex and dried seeds were proved to have antibacterial activity (Grosvenor et al., 1995). Kupchan et al. (1970) reported antileukemia and antinasopharyngeal agent jatrophone from J. elliptica. Jatropha leaves, which show antileukemic activity, contain α-amyrin, β-sitosterol, stigmasterol, campesterol, 7-keto-β-sitosterol and stigmast-5-ene-3-β-7-β-diol (Morton, 1981). Anticancer agents reported are jatropham and jatrophone from J. tanjorensis (2001). A new antibiotic agent R (+) 4-hydroxy-2-pyrrolidinone was isolated from the seed oil of J. curcas (Subramanian, 2005). 5.0. 2005). Further, antilesiocytic activity was higher than the standard vincristin sulphate 0.27 µg/ml. The LC50 was 4.67µg/ml, which was found to be more susceptible with an IC50 value of 43.24 µg/ml in MTT assay. It was followed by B16F10 with an IC50 value of 49.60 µg/ml. In SRB assay, IC50 value of 40.24 µg/ml to Hep-2 and 43.25 µg/ml to B16F10. A549 and NRK cell line showed less activity as indicated by the relatively high IC50 value (Tables 1, 2; Figs. 1, 2). The results in the present study showed that the leaf extract in J. tanjorensis is cytotoxic in nature and may possess antitumor activity.

4.3 In vivo Cytotoxic Activity:

Lacy and O’Kennedy (2004) studied the coumarins and coumarin-related compounds to determine the therapeutic role in the treatment of cancer and reported the most exerted and potent inhibitory effect of genistein and esculetin on cell growth in comparison to other compounds. Balaji et al. (2009) studied the methanol fraction of J. curcas for its anti-metastatic activity using B16F10 melanoma cells in C57BL/6 mice. Simultaneous administration of methanol fraction at the doses of 100 and 200 mg/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 tumor bearing animals, as compared to the untreated control animals.

The present study was carried out to investigate and evaluate the effect of methanol fraction of J. tanjorensis against B16F10, a highly metastatic melanoma cell line-induced metastasis in mice. The methanol fraction was found to be positive for the presence of flavonoids and alkaloids (Trease and Evans, 1996; Harborne, 1998). So, the methanol fraction and its isolated alkaloid R (+) 4-hydroxy-2-pyrrolidinone was used. In vivo study, the effect of methanol fractions (100 and 200 mg/kg) in J. tanjorensis on lung melanoma of B16F10 melanoma cells and survival of animals (in days) was 53.46 ± 3.4 (42.59% ILS-Increase in life span): 60.24 ± 4.2 (61.08% ILS). R (+) 4-hydroxy-2-pyrrolidinone at 10 mg/kg produced 62.12 ± 7.6 (66.00% ILS) (Table 3; Fig. 3). The present study showed that R (+) 4-hydroxy-2-pyrrolidinone exhibited relatively high increase in life span than methanol fraction. B16F10 melanoma cells are highly metastatic and form colonies of tumor nodules in the lungs when administered through tail vein, which in turn promote lung fibrosis and collagen deposition. In the earliest post-injection 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 tumor nodules and none of the other tissues show the establishment of tumors (Fidler, 1970). In the present study, the anti-metastatic activity of R (+) 4-hydroxy-2-pyrrolidinone and methanol fraction of J. tanjorensis in B16F10 melanoma-induced lung metastasis was analyzed. Oral administration of methanol...
extract at 100 and 200 mg/kg and R (+) 4-hydroxy-2-pyrrolidinone p.o. resulted in marked reduction in the metastasis by B16F10 melanoma (Table 3; Fig. 3). 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 extra-cellular matrix, especially collagen, will reduce the pulmonary function. There was an elevated level of lung collagen hydroxyproline content in metastasis bearing animals. Treatment with methanol extract at 100 and 200 mg/kg and R (+) 4-hydroxy-2-pyrrolidinone p.o. caused significant reduction in the accumulation of collagen and number of lung tumor nodules in the treated groups. These results are correlated with the inhibition of lung tumor nodules and the increase in life span of metastatic tumor bearing animals when treated with R (+) 4-hydroxy-2-pyrrolidinone and methanol fraction of J. tanjorensis. Previously (R) 5-hydroxy–pyrrolidin-2-one isolated from J. curcas leaves and showed anti-inflammatory activity in carrageenan-induced rat paw edema. In the present study, R (+) 4-hydroxy-2-pyrrolidinone exhibited cytotoxic activity both in vitro and in vivo studies and provides scientific evidence.

V. CONCLUSION

5.1 Cytotoxic Activity:

5.1.1 In vitro Cytotoxic Activity:

Methanol extract and R (+) 4-hydroxy-2-pyrrolidinone exhibit comparatively more cytotoxic activity against all the four cell lines such as Hep-2, B16F10, A549 and NRK.

5.1.2 In vivo Cytotoxic Activity:

R (+) 4-hydroxy-2-pyrrolidinone exhibits relatively high increase in life span than methanol fraction.

ACKNOWLEDGEMENT

We thank our management Dr. K.Sridharan, Chairman, Kasalasingam group of Institutions for their kind encouragement.

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