ISSN:0975-1459
Journal of Pharmaceutical Sciences and Research
www.jpsr.pharmainfo.in

Anti cancer activity of *n*-butanol extract of marine sediment fungi *Aspergillus protuberus sp1* against dalton's ascitic lymphoma in mice

S. Mathan[1], A. Anton Smith[2]*, J. Kumaran[3] and S. Prakash[4]

[1] *Department of Pharmacy, Karpagam University, Coimbatore, Tamil Nadu, India.*

[2] *Department of Pharmacy, Annamalai University, Annamalai nager - 608 002, Tamil Nadu, India.*

[3] *P.S. College of Nursing, Thalakulam, Kanyakumari District – 629 802, Tamil Nadu, India.*

[4] *Department of Biotechnology, Udaya School of Engineering, Ammandivilai Post, Kanyakumari District – 629204, Tamil Nadu, India.*

Abstract:
Marine derived fungi provide plenty of structurally unique and biologically active secondary metabolites. In our previous study, we screened the marine sediments collected from the different coastal locations of Kanyakumari district, south India. In this study, we identified a new fungal species *Aspergillus protuberus SP1* (Gen Bank Accession: HQ 386016). The n-butanol exogenous extract of *A.protuberus SP1* showed *in vitro* antibacterial and anticancer activity. This extract was subjected to column chromatographic separation using different solvent compositions in the ratio of 9:1 to 1:9 in each composition. The ethanol: methanol (9:1) composition was selected for GC-MS study, indicated the presence of nine compounds. The n- butanol extract was subjected to toxicity evaluation. The results showed that the ED_{50} of the extract is 200mg/kg body weight and this extract was safe and it did not induce any sub acute or chronic toxicities in the experimental animals. The present study was designed to evaluate the anticancer activity of n-butanol extract in cancer induced male Swiss albino mice. Dalton's Lymphoma Ascites (DLA) cells were used for the cancer induction in animals. Two doses of n-butanol extract 200 mg and 400 mg/kg body weight was utilized for the evaluation. The fluorouracil injection dose of 20 mg/kg body weight was used for the comparative evaluation. Body weight, life span, cell count, hematological and biochemical parameters of treated animals were analyzed and compared. The results strongly support the antitumor and hepatoprotective nature of n-butanol exogenous extract of *A.protuberus SP1*.
Keywords: *Aspergillus protuberus SP1*, n-butanol exogenous extract, anticancer activity, DLA cells, Swiss albino mice.

INTRODUCTION:

In the biosphere, ocean is the unique source of drug origin and many important classes of organisms have originated in this environment. The ocean occupies approximately 70% of the earth's surface and 80% of the animal species resides in this continuous territory due to its immense source of pharmaceuticals, food, mineral and energy [1]. In the recent years, a renaissance has occurred in marine pharmacology. Complex and highly chiral structures have been optimized by high salt concentrations and high pressure environments over millions of years, which confers marine organisms the potential to produce valuable therapeutic entities [2]. The search for new biomedical from marine organisms resulted in the isolation of approximately 10000 metabolites, many of which showed pharmacological properties. A broad spectrum of biological activities has been detected, such as antibiotic, antifungal, toxic, cytotoxic, neurotoxic, antimitotic, antiviral and antineoplastic. In recent years, new targets have been added to the general screening, e.g. AIDS, immunosuppression, anti-inflammation, Alzheimer disease, ageing processes and some tropical diseases. In 1989, UN National Cancer Research Institute announced that substances from blue green algae are active against AIDS and oncogenic viruses. Cancer has become an increasing public health problem due to its high rates of morbidity and mortality [3].

Marine derived fungi have been rich sources of structurally novel and biologically active secondary metabolites, which have become attractive as important resources for new chemicals in drug discovery [4-5]. In the oceans, fungi live as saprophytes, parasites and symbionts on various matrices such as sea, sand, logs, water, soil bubbles as well as algae and other microorganism [6]. Marine fungi have attracted great attention as considerable resources only since the late 1980. Furthermore it was reported that the corresponding chemistry of marine fungi was structurally diverse and related to that of terrestrial fungi [7]. In the last decade, there has been dramatic increase in the number of preclinical anticancer lead compounds extracted from metabolites of marine derived fungi [2].

In our previous study, we screened the marine sediments of south Indian coastal belt of Muttom, Kadiapattinam and Colachel. In this study, we identified a new fungal species *Aspergillus protuberus SP1* from the sediment of muttom coast (Gen Bank Accession: HQ 386016). The n-butanol exogenous extract of *A.protuberus SP1* showed antibacterial and anticancer activity. This extract was subjected to column chromatographic separation using different solvent compositions in the ratio of 9:1 to 1:9 in each composition. The ethanol: methanol (9:1) composition was selected for GC-MS study, indicated the presence of nine compounds [8].

In the toxicity evaluation it was found that the n-butanol extract is safe and it did not induce any sub acute or chronic toxicities in the experimental animals [9]. The ED_{50} of this extract was determined as 200mg/kg. The present study was designed to evaluate the anticancer activity of n-butanol extract against cancer induced male Swiss albino mice, is an attempt to provide a direction for further research.

MATERIALS AND METHODS:

Fungal extract preparation:

150 ml of Potato Dextrose Broth was prepared in a conical flask by using sea water and distilled water mixture in the ratio of 1:1 and sterilized by autoclaving. The fungus *Aspergillus protuberus SP1* was inoculated aseptically and incubated at 28°C with constant shaking. After three days of incubation, 100 ml of culture broth was taken and mixed with 50 ml of n-butanol and subjected to constant shaking for 12 hours. Then the solvent layer was separated by using separating funnel and preserved for experiments.

Animals:

Healthy young adult male Swiss albino mice weighing 20- 25 g were used for the experiments. They were obtained from the Central animal house, K.M College of Pharmacy, Madurai, Tamil Nadu, India. They were housed in standard environmental conditions like ambient temperature (25 ± 1°c), relative humidity (55 ± 5%) and 12 hour light / dark cycle. The animals were fed with standard pellet diet and water *ad libitum.* All animal experiments were carried out in accordance with the guidelines of committee for purpose of control and supervision on experiments on animals (OECD 423). The approval from institute animal ethical committee has obtained for conducting animal experiments (IAEC/KMCP/29).

Tumor cell line:

Dalton's lymphoma ascites (DLA) cells were obtained from Amala cancer research center, Trissur, Kerala, India. The cells maintained *in vivo* in Swiss albino mice by intraperitoneal transplantation. While transforming the tumor cells to the grouped animal the DLA cells were aspirated from peritoneal cavity of the mice using saline. The cell count was done and further dilutions were made to adjust the total cells to 1 x 10^6 cells/ ml. The final diluted solution was given by intraperitoneal injection.

Treatment protocol:

Swiss Albino mice were divided in to five group of five each. All the animals in four groups were injected with DLA cells (1 x 10^6 cells / mouse) intraperitoneally, and the remaining one group is normal control group.

Group 1 served as the normal control. Group 2 served as the tumor control. Group 1 and 2 receives normal diet and Water. Group 3 served as the positive control, which was treated with injection fluorouracil at 20mg/kg body weight, intraperitoneally. [10]. Group 4 served as treatment control received 200mg/kg of *n*-butanol extract of *Aspergillus protuberus SP1* administered through orally. Group 5 served as treatment control received 400mg/kg of n-butanol extract of *Aspergillus protuberus SP1* administered through orally

Treatment:

In this study drug treatment was given, once daily for 14 days. After the last dose all mice from each group were sacrificed and the blood was withdrawn from each mouse by retro orbital puncture bleeding and utilized for the analysis of clinical parameters such as cancer cell count, hematological and biochemical parameters.

Derived parameters:

Body weight:

All the mice were weighed, from the beginning to 15th day of the study. Average increase in body weight on the 15th day was determined.

Percentage increase in life span (ILS):

Survival time of treated groups were compared with those of control using the formula

$$\%\text{ILS} = \frac{\text{Life span of treated group}}{\text{Life span of control group}} - 1 \text{ x } 100$$

Clinical parameters:

Cancer cell count:

The ascetic fluid (0.1ml) from the peritoneal cavity of each mouse was withdrawn by sterile syringe and diluted with 0.8 ml of ice cold normal saline and 0.1 ml of tryphan blue (0.1mg /ml) and total number of the living cells were counted using hemocytometer.

Cell count = No. of cells x dilution / Area x thickness of liquid film

Hematological parameters:

The collected blood was analyzed for WBC, RBC, hemoglobin, platelets count and packed cell volume. These investigations were carried out in COBAS MICROS OT 18 Roche, Switzerland.

Biochemical parameters:

The serum was analyzed for aspartate amino transferase (AST), alanine amino transferase (ALT), alkaline phosphatase (ALP), total cholesterol (TC) and triglyceride (TG) levels. All biochemical investigations were done by using COBAS MIRA PLUS – S auto analyzer Roche, Switzer land and MAX MAT.

RESULTS AND DISCUSSION:

Effect on body weight, life span and cell count:

In the body weight analysis, the animals treated with n-butanol extract showed significant reduction in body weight comparing with tumor control animals. In case of average life span, the tumor control animals showed the life span of 49% whereas in group 4 and 5, animals treated with the n-butanol extract at the dose of 200mg and 400mg/kg body weight showed the life span of 75% and 79% respectively. The animal group treated with fluorouracil showed the life span of 93%. Regarding with cell count, the animals treated with n-butanol extract showed a significant reduction in the viable tumor cell count comparing with tumor control group animals (Table 1).

Effect on hematological parameters:

The hematological parameters such as WBC, RBC and platelet count, hemoglobin level and packed cell volume were analyzed. In this, the level of RBC, hemoglobin and platelets

were decreased and WBC count was significantly increased in the tumor control group comparing with normal control group. The treatment control group received n-butanol extract at 200 mg and 400 mg/kg dose showed a significant increase in RBC, hemoglobin, packed cell volume and platelets and significant reduction in WBC count comparing with positive control group received fluorouracil, which showed a better result in all these parameters (Table 2).

Effect on biochemical parameters:

The biochemical parameters such as total cholesterol, triglycerides, aspartate amino transferase, and alkaline phosphatase are increased in tumor control group animals comparing with normal control group animals. The treatment with *n*-butanol extract of *Aspergillus protuberus SP1* at a dose of 200 mg/kg and 400 mg/kg body weight reversed these changes towards the normal level. The treatment with standard 5- FU also gave similar results (Table 3).

Table 1: Effect of *n*-butanol extract of *Aspergillus protuberus SP1* on the life span, body weight and cancer cell count

Animal Group	*No. of animals*	*% ILS Life span*	*Body weight (g)*	*cell count (ml X 10^6)*
G_1	6	>>30 days	2.25±0.60	-
G_2	6	49%	7.82±0.98^{a**}	2.68±0.35^{a**}
G_3	6	93%	3.80±0.68^{b**}	1.40±0.20^{b**}
G_4	6	75%	4.20±0.86^{b**}	1.85±0.26^{b**}
G_5	6	79%	4.35±0.82^{b**}	1.78±0.21^{b**}

G_1 – Normal control; G_2 – Tumor control; G_3 – Positive control; G_4 – Treatment control (low dose); G5 – Treatment control (High dose).

All values are expressed as mean ± SEM for 6 animals in each group.

^{a}** – significantly different from control (G_1) at $P < 0.01$

^{b}** – significantly different from cancer control (G_2) at $P < 0.01$

Table 2: Effect of *n*-butanol extract of *Aspergillus protuberus SP1* on hematological parameters

Animal Group	*WBC (Cells/ml x10^3)*	*RBC Count (Mill/cumm)*	*Hemoglobin (Gm/dl)*	*Packed cell volume (%)*	*Platelets (Lakhs/cumm)*
G1	10.35 ±1.52	4.45±0.90	12.40 ±1.28	30.35±3.20	3.40±0.85
G2	14.20 ±2.35^{a**}	2.40±0.45^{a**}	7.30 ±0.90^{a*}	14.26±2.40^{a**}	1.70±0.60^{a**}
G3	11.30 ±1.80^{b**}	4.05±0.85^{b**}	11.6 ±1.52^{b**}	24.30±2.40^{b**}	2.82±0.95^{b**}
G4	12.55 ±1.85^{b**}	3.30±0.65^{b**}	10.55±1.30^{b**}	18.30±1.45^{b**}	2.10 ±0.72^{b**}
G5	12.10±1.60^{b**}	3.05±0.50^{b**}	10.22±0.96^{b**}	22.36±1.50^{b**}	2.35±0.90^{b**}

G_1 – Normal control; G_2 – Tumor control; G_3 – Positive control; G_4 – Treatment control (low dose); G5 – Treatment control (High dose)

All values are expressed as mean ± SEM for 6 animals in each group.

^{a}** – Values are significantly different from control (G_1) at $P < 0.01$

^{b}** – Values are significantly different from cancer control (G_2) at $P < 0.01$

Table 3: Effect of *n*-butanol extract of *Aspergillus protuberus SP1* on biochemical parameters

Animal Group	*Cholesterol (mg/dl)*	*TGL (mg /dl)*	*AST (U/L)*	*ALT (U/L)*	*ALP (U/L)*
G_1	112.25±3.65	134.95±2.50	41.60 ±1.30	34.45 ±1.50	127.35 ±2.40
G_2	145.92±4.60^{a**}	222.35±4.80^{a**}	88.5±2.68^{a**}	62.40±2.65^{a**}	245.40±4.30^{a**}
G_3	122.45±3.82^{b**}	163.60±2.40^{b**}	56.40 ±1.80^{b**}	43.42±1.80^{b**}	162.42±2.50^{b**}
G_4	123.45±3.65^{b**}	178.62±2.60^{b**}	68.40±1.90^{b**}	49.30 ±1.95^{b**}	194.60±2.62^{b**}
G_5	120.30±3.45^{b**}	170.80±2.53^{b**}	62.60 ±2.30^{b**}	46.25±1.70^{b**}	190.50±2.30^{b**}

G_1 – Normal control; G_2 – Tumor control; G_3 – Positive control; G_4 – Treatment control (low dose); G5 – Treatment control (High dose)

In our previous study, a new fungus *A.protuberus SP1* was identified from the marine sediments collected from the Muttom coast of Kanyakumari district, Tamil nadu, south India. It showed significant antibacterial activity. Among the different exogenous and endogenous extract of this fungus, the n-butanol exogenous extract showed significant effect in *in vitro* antibacterial and anticancer activity. The presence of nine compounds in the extract was confirmed by GC-MS evaluation [8]. In the toxicity evaluation it was found that the Ed_{50} of the n-butanol extract is 200 mg/kg and the extract is safe and it did not induce any sub acute or chronic toxicities in the experimental animals. In the present study the n-butanol extract was subjected to anticancer evaluation in the tumor induced Swiss albino mice. Two doses of extract 200 mg and 400 mg/kg body weight were utilized in the study. Fluorouracil injection at the dose of 20 mg/kg body weight

was given to one group of tumor induced animals for comparative evaluation. In case of body weight analysis the animals treated with n-butanol extract showed significant reduction in body weight comparing with tumor control animals. In case of average life span, the tumor control animals showed the life span of 49% whereas, animals treated with the n-butanol extract at the dose of 200mg and 400mg/kg body weight showed the life span of 75% and 79% respectively. These values were highly significant, however the average life span of Fluorouracil treatment was found to be 93%, indicating its potent antitumor nature. The reduction in the viable tumor cell count and changes in hematological and biochemical parameters also support the antitumor nature of the n-butanol exogenous extract of *A.protuberus SP1*. From these studies, it is clear that further works needed to be done in the future for the development of clinically useful chemotherapeutic agents.

CONCLUSION:

In summary, marine derived fungi provide plenty of structurally unique and biologically active secondary metabolites. In our previous study, a new fungus *A.protuberus SP1* was identified from the marine sediments collected from the Muttom coast of Kanyakumari District, Tamil nadu, south India. Among the different extracts, the n-butanol exogenous extract of this fungus showed significant antibacterial and anticancer activity. In the GC-MS study, the extract showed the presence of nine compounds. (z,z)–9,12 Octadecadienoic acid, one among them having broad spectrum biological activities including anticancer activity as per previous literatures. In the toxicity evaluation it was found that the n-butanol extract is safe and it did not induce any sub acute or chronic toxicities in the experimental animals. Now in the present study, this extract showed a significant anticancer and hepato protective effect in the tumor induced male Swiss albino mice. This study could emerge a new specific leads from marine fungal metabolites to target the various cancer cells.

REFERENCES:

[1] Prakash, S., Valentin Bhimba, B., *Nat Prod Rad.* 2005, 4 (4), 264 - 269.

[2] Li- yang Tao, Jian-Ye Zhang, Yong-ju Liang, Li-Ming Chen, Li-sheng Zheng, Fang Wang, Yan–jun Mi, Zhi-gang She, Kenneth Kin Wah To, Yong- cheng Lin Li-wu Fu., *Mar.Drugs.* 2010, 8, 1094 – 1105.

[3] Ely, R., Supriya, T., Naik , CG., *J. Exp. Biol. Ecol.* 2004, 309 (1), 121-127.

[4] Rejeev Kumar Jha, Xu Zi-rong., *Mar. Drugs.* 2004, 2(3), 123 - 146.

[5] Molinski , TF., Dalisay, DS., Lievens , SL., Saludes, JP., *Nat .Rev. Drug Discov.* 2009, 8, 69 - 85.

[6] Mansuma, R., Yamaguchi, Y., Novmi, M., Omura, S., Namikoshi, M., *Mycoscience*. 2001, 24(5), 455-459.

[7] Samuel, P., Prince, L., Prabakaran, P., *J. Microbiol. Biotech. Res*. 2011, 1(4), 86-94.

[8] Mathan, S., Anton Smith, A., Kumaran, J., Prakash, S., *Chin. J. Nat. Med.* 2011, 9 (4), 286 – 292.

[9] Mathan, S., Anton Smith, A., Kumaran, J., Prakash, S., *Chin. J. Nat. Med.* (In Press).

[10] Sathiyanarayanan, L., Shinnathambi, Arulmozi, Chidhambarnathan, N., *Iran. J. Pharmacol. Toxicol.*, 2006, 6, 133 – 136.