Research Article

CHRONIC AND SUB ACUTE TOXICITY STUDY OF THE N-BUTANOL EXTRACT OF THE MARINE SEDIMENT FUNGUS *ASPERGILLUS PROTUBERUS* sp1

S. Mathan ¹, A. Anton Smith ², Shaiju. S. Daran¹, J. Kumaran ^{3*}, S. Prakash ⁴

¹Ezhuthachan College of Pharmaceutical Sciences, Neyattinkara, Thiruvananthapuram, Kerala, India

²Department of Pharmacy, Annamalai University, Annamalai nager, Tamil Nadu, India

³P.S. College of Nursing, Thalikulam, Kanyakumari District, Tamil Nadu, India

⁴Research Director, Cherraan's Institute of Health Sciences, Coimbatore, Tamil Nadu, India

*Corresponding Author Email: kumaranjvkm@yahoo.co.in

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ABSTRACT

This study was designed to evaluate the toxicity of the n- butanol extract of marine derived fungi *Aspergillus protuberus* sp1. Lethal dose (LD₅₀), chronic and sub acute toxicities were determined in the rats. From the LD₅₀, the effective dose (ED₅₀) was determined. The n-butanol extract was prepared at the concentrations of 200, 400, and 600 mg.kg⁻¹ body weight. In the sub acute toxicity study, the prepared extract was given orally to the experimental rats for fourteen days and in case of chronic toxicity study, it was given for ninety days. Then the animals were observed for the biochemical and hematological effects, macroscopic and microscopic changes in the liver and kidney. Results showed that, in both sub acute and chronic toxicity studies, the experimental rats showed normal general behavior, respiratory pattern, cardiovascular signs, motor activities, reflexes and normal change in skin and fur. The results also showed that the extract did not induce any biochemical, hematological effects, macroscopic and microscopic changes in the liver and kidney. Collectively, these data demonstrate that the extract is safe and these results are useful for further investigation of this fungus in the future.

Keywords: *Aspergillus protuberus* sp1, LD₅₀, Sub acute toxicity, Chronic toxicity

INTRODUCTION

In the biosphere, the 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 reside in this continuous territory due to its immense source of pharmaceuticals, food, minerals and energy¹. In the recent years, a renaissance has occurred in marine pharmacology. Complex and highly chiral structures have been optimized by the high salt concentrations and the high pressure environments over millions of years, which confers marine organisms the potential to produce valuable therapeutic entities². The search for new bio medical compounds from marine organisms has resulted in the isolation of approximately 10,000 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, the US 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 mortality³.

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 microorganisms⁶. Marine fungi

have attracted great attention as considerable resources only since the late 1980s. Furthermore, it was reported that the corresponding chemistry of marine fungi was structurally diverse and related to that of terrestrial fungi⁷. In the last decade, there has been a dramatic increase in the number of preclinical anticancer lead compounds extracted from metabolites of marine derived fungi². Toxicity evaluation of lead compounds is one of the important steps in drug development. Toxicity is defined as any harmful effect of a chemical or a drug on a target organism. Acute, sub acute and chronic toxicities have been defined by various experts. The Organisation for Economic Co-operation and Development panel of experts (OECD Guidelines, 1980) defines acute toxicity as the adverse effects occurring within a short time of administration of a single dose of a substance or multiple doses given with in twenty four hours^{8,9}. Sub acute toxicity is defined as the adverse effects occurring as a result of the repeated daily dosing of a chemical to experimental animals for fourteen days⁹ and chronic toxicity as the adverse effects occurring as a result of the repeated daily dosing of chemical to experimental animals for ninety days.

In a previous study, in this laboratory, the marine sediments of south Indian coastal belt of Muttom, Kadiapattinam and Colachel were screened. In this study, a new fungal species *Aspergillus protuberus* sp1 from sediment of Muttom coast (Gen Bank Accession: HQ 386016) was identified. 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, and indicated the presence of nine compounds¹⁰. The present study was designed to evaluate the toxicity of the n-

butanol extract, an attempt to provide a direction for further research.

MATERIALS AND METHODS

Fungal extract preparation

Potato Dextrose Broth (150 ml) was prepared in a conical flask by using a seawater 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 incubation, a sample (100 ml) of the culture broth was taken and mixed with n-butanol (50 ml) and subjected to constant shaking for 12 h. Then the solvent layer was separated and preserved for experiments.

Animals

Healthy young adult Sprague - Dawley rats of both sexes, eight to ten weeks old, were used for the experiments. They were obtained from the Central Animal House, K.M College of Pharmacy, Madurai, Tamil Nadu, India. All of the animal experiments were carried out in accordance with the guidelines of the Committee for Purpose of Control and Supervision on Experiments on Animals. The institute animal ethical committee has approved the conduct for the animal experiments (IAEC/KMCP/28).

Determination of LD₅₀

LD₅₀ is the dose that is lethal to 50 % of the test animal population. LD₅₀ of the extract was determined as per the guidelines of Organization of Economic Co-Operation and Development (OECD 2001).

Randomly selected animals were marked enabling identification and kept for five days prior to dosing to allow for acclimatization to the laboratory conditions. A temperature of 22±3°C temperature was maintained in the experimental room. Conventional laboratory diet with unlimited drinking water was provided for feeding. The test substance was administered in a single dose by gavage using stomach tube. The starting dose was selected as 50 mg.kg⁻¹ body weight. This dose was administered to three animals. The number of animal deaths was noted after 24 h. After that the next dose 300 mg.kg⁻¹ was attempted. After 24 hr the number of animal deaths was noted. Then the final dose 2000 mg.kg⁻¹ was administered and the animal death was noted.

Sub acute toxicity study

Sub acute toxicity study was carried out according to the guidelines of World Health Organization (WHO 2000) and the Organization of Economic Co-operation and Development (OECD 2001). Selected animals were randomly divided in to four groups of six each. They were housed under controlled environment (temp. 22±3°C and 12 h dark- light cycle) and allowed free access to drinking water and standard pellet diet produced by Hindustan Rat Chow Feed Group Co. Ltd. Rats were deprived of food except water 16 -18 h prior to the experiments. Using distilled water, the n-butanol extract of *A. protuberus* sp1 was prepared at the concentrations of 200, 400, and 600 mg.kg⁻¹ body weight. The prepared extract was given orally for 14 day, while the control group received water vehicle. Toxic manifestations such as body weight, signs of toxicity, and mortality were observed daily. At the end of study, all rats were fasted over night and anaesthetized for blood collection. Heparinised blood samples were utilized for the determination of complete blood count, RBC count, platelet count, and red cell indices. The serum from non heparinised blood was carefully collected for blood chemistry and enzyme analysis. All rats were sacrificed after the blood collection. The liver and kidney were preserved in 10%

neutral buffered formalin solution for histopathological examination. Results are expressed as means ± Standard Error Mean (S.E.M). Statistical significance was determined by one way analysis of variance (ANOVA) followed by Newmann Keul's multiple range tests. *P* values less than 0.05 were considered significant.

Chronic toxicity study

The chronic toxicity study was carried out according to the guidelines of Organization of Economic Co-operation and Development for the testing of chemicals 452 (OECD2001). Selected animals were randomly divided in to four groups of six each. They were housed under a controlled environment (temp. 22±3°C and 12 h dark- light cycle) and allowed free access to drinking water and standard pellet diet produced by Hindustan Rat Chow Feed Group Co. Ltd. Rats were deprived of food, except water, 16 -18 h prior to the experiments. Using distilled water, the n-butanol extract of *A. protuberus* sp1 was prepared at the concentration of 200, 400, and 600 mg.kg⁻¹ body weight. The prepared extract was given orally for 90 days, while the control group received water vehicle. Potential manifestations of toxicity such as body weight, signs of toxicity, and mortality were observed daily. At the end of the study, all of the rats were fasted overnight, and anesthetized for blood collection. Heparinised blood samples were utilized for the determination of complete blood count, RBC count, Platelet count, and red cell indices. The serum from non heparinised blood was carefully collected for blood chemistry and enzyme analysis. All the rats were sacrificed after the blood collection. The internal organs and tissues were weighed to determine the relative organs weight and observed for gross lesions. All tissues were preserved in 10% neutral buffer formalin solution for histopathological examination. Results were expressed as means ± Standard Error Mean (S.E.M). Statistical significance (ANOVA) followed by Newmann Keul's multiple range tests. *P* values less than 0.05 were considered significant.

RESULTS

The n-butanol extract of *A. protuberus* sp1 was selected for toxicity evaluation. It was subjected to acute and chronic toxicity studies. Healthy young adult Sprague – Dawley rats of both sexes, eight to ten weeks old, were used for the experiments.

Determination of LD₅₀

The LD₅₀ of the extract was determined as per OECD guidelines. After administration of the first two doses, none of the animals died. However after the administration of final dose, of the three animals two had died within 24 h of administration. From this, the 2000mg/kg dose was concluded as the LD₅₀ and from this, the ED₅₀ was determined as 200mg/kg.

Sub acute toxicity study

In the sub-acute toxicity study, a total of 24 rats were used. They were divided into four groups of six each, and fed with drinking water and standard pellet diet produced by Hindustan Rat Chow Feed Group Co. Ltd. (Table 1).

Rats were deprived of food, except water, 16 -18 h prior to the experiments. The extract was prepared in three types of concentration such as 200, 400, and 600 mg.kg⁻¹ body weight using distilled water. Each concentration was given orally to each group of animals and the control group received water vehicle for 14 days.

Toxic manifestations, such as body weight, signs of toxicity, and mortality, were observed daily. At the end of the study biochemical evaluation was performed to evaluate the toxic effects on liver and

kidney. With respect to general behaviors, both male and female rats treated with the extract at all three doses had no signs of behavior changes and toxic symptoms. There were no significant body weight changes from the initial body weight of both male and female animals (Table 2). Both females and males rats were healthy, as shown by the normal appearance of general behavior, respiratory pattern, cardiovascular signs, motor activities, and normal changes in skin and fur. In regard to hematological findings, most of the values in the treated groups were normal in comparison with the control group (Table 3). Therefore, these results suggest that the extract did not cause hematological or immunological effects in rats. In case of bio-chemical parameters, the results showed minor changes, but remained within the normal range (Table 4). In the histopathological evaluation of the liver, all three treated groups showed no significant macroscopic and microscopic changes in comparison with control. The evaluation of kidney also showed no significant changes.

Chronic toxicity study

In the chronic toxicity study, another twenty four rats were used. They were divided into four groups of six rats each, and fed with drinking water and standard pellet diet produced by Hindustan Rat Chow Feed Group Co. Ltd.

Rats were deprived of food, except water, 16 -18 h prior to the experiments. The extract was prepared in three types of concentration such as 200, 400, and 600 mg.kg⁻¹ body weight using distilled water. Each concentration was given orally to each group of animals and the control group received water vehicle for 90 days. Toxic manifestations, such as body weight, signs of toxicity, and mortality were observed daily. At the end of the study, biochemical evaluation was performed to evaluate the toxic effects on the liver and kidney. With respect to general behaviors, both male and female

rats treated with the extract at all three doses had no signs of behavior changes and toxic signs. The female treated groups showed a slight increase of body weights, but were not significantly different from those of control. However, the male treated groups showed a slight increase in the body weight and body weight gain in comparison with the control group (Table 5). The effects of body weight and body weight gain may have resulted from physiological changes in the rats such as metabolism, and food and water intake. The results from animal health monitoring over the entire period of 90 days showed no sign of morbidity or diseases. Both female and male rats were healthy, as shown by the normal appearance of general behavior, respiratory pattern, cardiovascular signs, motor activities, reflexes, and normal change in skin and fur. In regard to hematological evaluation, most of the findings in the treated groups were normal in comparison with the control group. Significantly, some values were different from those of the control group such as red blood cells, mean corpuscular volume, mean corpuscular hemoglobin, and platelet. However, such values are within the normal ranges (Table 6). These variations may have resulted from normal variation among animal groups. Therefore, these results suggest that the extract did not cause hematological or immunological defects in rats. In the biochemical findings, the results showed minor alterations but remain within the normal range (Table 7). In the histopathological evaluation, the liver of treated groups showed no macroscopic and microscopic changes in comparison with the control group. The section showed normal liver architecture. Central vein, hepatocytes, and portal tracts appeared normal. The evaluation of the kidney also showed no significant changes. The section showed renal parenchyma with renal tubules and glomeruli appearing normal. In the organ weight findings, the male and female treated groups showed no significant differences compared with the control group (Table 8).

Table 1: Food consumption (g / rat / day) of rats in sub-acute toxicity study

Group	Day-0	Day-14
	Male	
Control	18.2	19.5
200 mg.kg ⁻¹	19.6	23.4
400 mg.kg ⁻¹	20.9	25.3
600 mg.kg ⁻¹	22.3	26.7
	Female	
Control	17.5	18.2
200 mg.kg ⁻¹	20.7	21.3
400 mg.kg ⁻¹	23.8	24.4
600 mg.kg ⁻¹	22.1	25.2

Table 2: Body weight changes of rats in sub-acute toxicity study

Group	Initial B.W (g)	B.W after treatment (g)
	Male	
Control	220.50 ± 10.5	253.15 ± 11.8
200 mg.kg ⁻¹	216.20 ± 9.2	249.25 ± 11.4
400 mg.kg ⁻¹	210.75 ± 8.6	246.10 ± 11.0
600 mg.kg ⁻¹	225.15 ± 10.9	249.85 ± 11.6
	Female	
Control	212.30 ± 8.2	248.20 ± 10.4
200 mg.kg ⁻¹	210.15 ± 9.0	240.18 ± 12.2
400 mg.kg ⁻¹	222.40 ± 10.2	256.05 ± 10.6
600 mg.kg ⁻¹	219.22 ± 9.2	248.19 ± 11.5

B.W – Body weight. Values are mean ± S.E.M (n = 6). All groups were treated with oral dose of 200, 400, and 600 mg.kg⁻¹ bodyweight/day for 14 days. No significant body weight changes from initial body weight.

Table 3: Hematological findings in the sub-acute toxicity study

Parameter	Control	Dose of the extract (mg.kg ⁻¹)		
		200	400	600
Male				
R.B.C.	7.95 ± 0.17	8.05 ± 0.08	8.17 ± 0.13	8.21± 0.11
Haemoglobin (g.dL ⁻¹)	15.44 ± 0.33	15.10 ± 0.12	15.8 ± 0.17	15.2 ± 0.09
M.C.V.	59.44 ± 0.89	58.77 ± 0.35	59.45 ± 0.43	58.92 ± 0.23
M.C.H.C.	32.45 ± 0.23	31.98 ± 0.23	32.34 ± 0.13	32.45 ± 0.23
Platelet (× 10 ⁵ /μL)	5.55 ± 0.32	5.43 ± 0.20	5.22 ± 0.26	5.40 ± 0.24
W.B.C.	3.65 ± 0.32	4.12 ± 0.12	4.28 ± 0.25	4.44 ± 0.20
Female				
R.B.C.	7.39 ± 0.17	7.69 ± 0.18	7.27 ± 0.33	7.30 ± 0.21
Haemoglobin (g.dL ⁻¹)	15.44 ± 0.23	15.9 ± 0.32	15.23 ± 0.20	15.36 ± 0.13
M.C.V.	60.44 ± 0.69	61.73 ± 0.25	60.45 ± 0.33	61.92 ± 0.1
M.C.H.C.	33.48 ± 0.15	34.98 ± 0.26	33.34 ± 0.16	34.45 ± 0.19
Platelet (× 10 ⁵ /μL)	5.68 ± 0.19	5.24 ± 0.22	5.12 ± 0.28	5.35 ± 0.35
W.B.C	2.65 ± 0.26	2.87 ± 0.15	2.98 ± 0.21	3.24 ± 0.24

R.B.C – Red blood cell (× 10⁶/μL). M.C.V – Mean corpuscular volume (fl). M.C.H.C – Mean corpuscular hemoglobin concentration (g/dl). W.B.C – White blood cells (× 10³/μL). Values are mean ± S.E.M (n = 6). All groups were treated with oral dose of 200, 400, and 600 mg.kg⁻¹ bodyweight/day for 14 days. No significant difference from normal control.

Table 4: Biochemical findings in sub-acute toxicity study

Parameter	Control	Dose of the extract (mg.kg ⁻¹)		
		200	400	600
Male				
Glucose (mg.dL ⁻¹)	146.80 ± 8.34	133.27 ± 2.81	148.35 ± 0.36	153.76 ± 3.87
BUN (mg.dL ⁻¹)	21.34 ± 1.32	19.35 ± 0.78	19.56 ± 0.95	20.45 ± 0.36
Creatinine (mg.dL ⁻¹)	0.41 ± 0.01	0.39 ± 0.03	0.40 ± 0.02	0.38 ± 0.03
Total Protein (g.dL ⁻¹)	5.65 ± 0.13	5.78 ± 0.09	5.84 ± 0.12	5.98 ± 0.15
Albumin (g.dL ⁻¹)	3.78 ± 0.05	3.83 ± 0.07	3.88 ± 0.04	3.92 ± 0.05
Total Bilirubin (mg.dL ⁻¹)	0.14 ± 0.02	0.11 ± 0.01	0.13 ± 0.01	0.15 ± 0.02
AST (U.l ⁻¹)	139.24 ± 15.23	128.34 ± 16.10	133.54 ± 19.32	135.35 ± 21.25
ALT (U.l ⁻¹)	80.25 ± 24.08	65.3 ± 19.34	73.48 ± 18.92	78.76 ± 17.56
ALP (U.l ⁻¹)	68.27 ± 10.24	55.38 ± 11.4	59.34 ± 8.36	62.36 ± 8.92
Female				
Glucose (mg.dL ⁻¹)	133.85 ± 4.27	128.32 ± 5.67	130.34 ± 8.56	132.46 ± 7.32
BUN (mg.dL ⁻¹)	18.34 ± 0.76	17.36 ± 0.43	17.54 ± 0.53	17.94 ± 0.34
Creatinine (mg.dL ⁻¹)	0.42 ± 0.03	0.44 ± 0.02	0.46 ± 0.05	0.45 ± 0.03
Total Protein (g.dL ⁻¹)	5.67 ± 0.13	5.73 ± 0.15	5.79 ± 0.18	5.82 ± 0.13
Albumin (g.dL ⁻¹)	4.18 ± 0.04	4.23 ± 0.07	4.32 ± 0.06	4.38 ± 0.08
Total Bilirubin (mg.dL ⁻¹)	0.17 ± 0.01	0.18 ± 0.01	0.19 ± 0.02	0.19 ± 0.01
AST (U.l ⁻¹)	137.14 ± 14.28	126.22 ± 16.18	132.34 ± 15.28	134.42 ± 23.21
ALT (U.l ⁻¹)	47.12 ± 5.87	44.34 ± 5.32	45.76 ± 5.92	46.32 ± 6.12
ALP (U.l ⁻¹)	34.28 ± 2.35	30.19 ± 2.78	32.72 ± 3.12	33.46 ± 2.46

BUN – Blood urea nitrogen. AST – Aspartate amino transferase. ALT – Alanine amino transferase. ALP – Alkaline phosphatase. Values are expressed as mean ± S.E.M (n=6). All groups were treated with oral dose of 200,400,600 mg.kg⁻¹ body weight/day for 14 days.

Table 5: Body weight changes of rats in the chronic toxicity study

Groups	Initial B.W (g)	B.W after treatment (g)
Male		
Control	218.42 ± 5.56	296.25 ± 8.70
200 mg.kg ⁻¹	222.35 ± 5.78	301.15 ± 8.16
400 mg.kg ⁻¹	226.70 ± 6.15	299.56 ± 8.85
600 mg.kg ⁻¹	232.22 ± 6.50	308.45 ± 8.80
Female		
Control	210.50 ± 6.32	265.65 ± 7.85
200 mg.kg ⁻¹	216.22 ± 5.25	275.05 ± 8.12
400 mg.kg ⁻¹	220.74 ± 6.40	288.12 ± 8.25
600 mg.kg ⁻¹	222.15 ± 6.78	271.22 ± 7.45

B.W – Body weight. Values are mean ± S.E.M (n = 6). All groups were treated with oral dose of 200, 400, and 600 mg.kg⁻¹ bodyweight/day for 90 days. In males, weight gain different from normal control at P < 0.05. In females, no significant difference from normal control was observed.

Table 6: Haematological findings in chronic toxicity study

Parameter	Control	Dose of the extract (mg.kg ⁻¹)		
		200	400	600
Male				
R.B.C.	7.66 ± 0.26	8.25 ± 0.12	8.36 ± 0.55	8.40 ± 0.36
Haemoglobin (g.dL ⁻¹)	14.23 ± 0.56	16.23 ± 0.16	16.1 ± 0.85	15.5 ± 0.12
M.C.V.	61.40 ± 0.88	57.80 ± 0.39	60.56 ± 0.54	62.50 ± 0.89
M.C.H.C.	30.56 ± 0.35	32.45 ± 0.55	36.45 ± 0.46	38.52 ± 0.75
Platelet (× 10 ⁵ /μL)	5.26 ± 0.40	5.56 ± 0.54	5.25 ± 0.40	5.85 ± 0.53
W.B.C.	3.60 ± 0.42	4.52 ± 0.42	4.42 ± 0.34	4.52 ± 0.30
Female				
R.B.C.	7.52 ± 0.36	7.72 ± 0.45	7.32 ± 0.36	7.35 ± 0.25
Haemoglobin (g.dL ⁻¹)	15.96 ± 0.75	16.1 ± 0.46	15.35 ± 0.27	15.45 ± 0.26
M.C.V.	67.55 ± 0.72	62.75 ± 0.45	61.56 ± 0.36	62.15 ± 0.32
M.C.H.C.	34.56 ± 0.45	35.95 ± 0.42	32.45 ± 0.32	32.88 ± 0.65
Platelet (× 10 ⁵ /μL)	5.75 ± 0.36	5.78 ± 0.52	5.25 ± 0.42	5.54 ± 0.56
W.B.C.	2.82 ± 0.53	2.96 ± 0.62	3.05 ± 0.28	3.28 ± 0.46

R.B.C – Red blood cell (× 10⁶/μL). M.C.V – Mean corpuscular volume (fl). M.C.H.C – Mean corpuscular hemoglobin concentration (g.dL⁻¹). W.B.C – White blood cells (× 10³/μL). Values are mean ± S.E.M (n = 6). All groups were treated with oral dose of 200, 400, and 600 mg.kg⁻¹ bodyweight/day for 90 days; No significant difference from normal control.

Table 7: Biochemical findings in chronic toxicity study

Parameter	Control	Dose of the extract (mg.kg ⁻¹)		
		200	400	600
Male				
Glucose (mg.dL ⁻¹)	150.15 ± 6.12	136.32 ± 2.65	144.56 ± 0.42	150.05 ± 5.96
BUN (mg.dL ⁻¹)	23.42 ± 2.56	20.40 ±1.80	19.23 ± 1.45	21.95 ± 1.53
Creatinine (mg.dL ⁻¹)	0.43 ± 0.06	0.38 ± 0.05	0.42 ± 0.08	0.40 ± 0.07
Total Protein (g.dL ⁻¹)	5.78 ± 0.68	5.85 ± 0.75	5.95 ± 0.82	6.05 ± 0.96
Albumin (g.dL ⁻¹)	3.82 ± 0.15	3.92 ± 0.23	4.05 ± 0.30	4.09 ± 0.38
Total Bilirubin (mg.dL ⁻¹)	0.16 ± 0.04	0.20 ± 0.10	0.18 ± 0.08	0.19 ± 0.09
AST (U.I ⁻¹)	142.35 ±15.42	135.45 ± 14.23	138.62 ± 15.45	140.42 ± 17.32
ALT (U.I ⁻¹)	82.32 ± 6.12	80.4 ± 6.22	78.52 ± 6.15	81.24 ± 5.30
ALP (U.I ⁻¹)	70.34 ± 5.45	65.21 ± 4.40	69.05 ± 4.35	68.78 ± 4.55
Female				
Glucose (mg.dL ⁻¹)	130.42 ± 5.56	135.65 ± 5.25	138.45 ± 5.60	145.52 ± 5.41
BUN (mg.dL ⁻¹)	20.15 ± 1.45	18.42 ± 1.55	19.86 ± 1.65	21.25 ± 1.42
Creatinine (mg.dL ⁻¹)	0.50 ± 0.10	0.40 ± 0.09	0.51 ± 0.15	0.48 ± 0.09
Total Protein (g.dL ⁻¹)	5.70 ±0.88	6.05 ± 0.95	6.18 ± 0.98	6.20 ± 0.90
Albumin (g.dL ⁻¹)	4.35 ± 0.45	4.58 ± 0.56	4.65 ± 0.68	4.70± 0.75
Total Bilirubin (mg.dL ⁻¹)	0.18 ± 0.02	0.20 ± 0.04	0.22 ± 0.08	0.30 ± 0.09
AST (U.I ⁻¹)	140.22 ± 13.30	130.30 ± 12.30	132.58 ± 12.32	130.60 ± 10.35
ALT (U.I ⁻¹)	49.35 ± 6.92	50.30 ± 6.45	51.80 ± 6.25	49.20 ± 6.68
ALP (U.I ⁻¹)	36.45 ± 3.45	32.35 ± 4.85	34.80 ± 4.57	38.50 ± 4.86

BUN – Blood urea nitrogen. AST – Aspartate amino transferase. ALT – Alanine amino transferase. ALP – Alkaline phosphatase. Values are expressed as mean $\pm$ S.E.M (n=6). All groups were treated with oral dose of 200,400,600 mg.kg⁻¹ body weight/day for 90 days

Table 8: Organ weight findings at the end of the chronic toxicity study

No	Organ	Control	Dose of the extract (mg.kg ⁻¹)		
			200	400	600
Male					
1	Brain	2.06 ± 0.03	1.96 ± 0.04	1.99 ± 0.06	2.10 ± 0.08
2	Lungs	2.20 ± 0.10	2.22 ± 0.14	2.30 ± 0.20	2.32 ± 0.22
3	Heart	1.83 ± 0.06	1.80 ± 0.07	1.90 ± 0.09	1.96 ± 1.10
4	Liver	16.20 ± 0.52	15.66 ± 0.40	15.17 ± 0.38	14.90± 0.35
5	Spleen	1.13 ± 0.07	1.06 ± 0.05	1.10 ± 0.08	1.11 ± 0.09
6	Kidneys	1.92 ± 0.07	1.86 ± 0.06	1.78 ± 0.05	1.90 ± 0.09
Female					
1	Brain	1.96 ± 0.02	1.92 ± 0.04	1.98 ± 0.06	2.05 ± 0.08
2	Lungs	1.85 ± 0.15	1.78 ± 0.10	1.89 ± 0.18	1.95 ± 0.20
3	Heart	1.20 ± 0.05	1.26 ± 0.06	1.28 ± 0.07	1.32 ± 0.08
4	Liver	9.88 ± 0.48	9.66 ± 0.44	10.05 ± 0.50	10.12 ± 0.54
5	Spleen	0.92 ± 0.04	0.90 ± 0.03	0.98 ± 0.06	1.02 ± 0.07
6	Kidneys	1.32 ± 0.06	1.29 ± 0.04	1.36 ± 0.08	1.40 ± 0.08

Values are expressed as mean $\pm$ S.E.M (n=6). Values are not significantly different from those of the control group.

DISCUSSION

In a previous study from this laboratory, 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 exogenous and endogenous extracts of this fungus, the n-butanol exogenous extract showed significant antibacterial and anti-cancer activity. In the GC-MS evaluation, the presence of nine compounds in the extract was confirmed¹⁰. But toxicity evaluation is essential to ensure the safety of any agent. Usually three types such as acute, subacute and chronic toxicity evaluations are carried out. Acute toxicity evaluation gives the information about LD₅₀. Both subacute and chronic evaluations give information about the cumulative toxicity at low dose on prolonged period of time¹¹. With this view, in the present study, the extract was subjected to a toxicity study. The results showed that the extract is safe, and it did not induce any sub-acute or chronic toxicity in the experimental animals. Importantly, (Z, Z) - 9, 12 octadecadienoic acid, having broad spectrum biological activities including anticancer activity^{12, 13}, is one of the compounds present in this extract according to the GC-MS study. From these studies, it is clear that further work is needed to be done in the future for the development of clinically useful chemotherapeutic agents.

CONCLUSION

In summary, marine derived fungi provide numerous structurally unique and biologically active secondary metabolites. The n-butanol exogenous extract of *A. protuberus* sp1 which had previously shown antibacterial and anticancer activity, did not induce any sub-acute or chronic toxicities in experimental animals. In the earlier 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. The study area- Muttom, Kadiyapattanam, Manavalakurichy and the Colachel coastal region of south India are rich with radioactive minerals. Due to the presence of radioactive minerals, people of this region are affected with various types of cancers as per the previous surveys. It is hoped that this study could provide new specific leads from marine fungal metabolites to target various cancer cells.

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