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Antitumor and Immunomodulatory Activity of *Phallusia nigra* Savigny, 1816 Against Ehrlich Ascites Carcinoma

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Abstract

Antitumor and Immunomodulatory activity of the ethanol extract of the simple ascidian Phallusia nigra was assessed against Ehrlich Ascites Carcinoma (EAC) cells. 100% toxicity was observed at a concentration of 0.40 mg/ml. The extract at 50, 100, 150 mg/kg body weight and standard drug Vincristin at 0.08 mg/kg was administered to different groups. The results showed significant reduction in weight of body, thymus, lungs and an increase in the weight of spleen, liver and kidney. There was a decrease in tumor volume, packed cell volume, viable cell count and increase in nonviable cell count and median survival time there by an increase in lifespan. Hematological parameters were restored to normal. The bone marrow cellularity and β esterase positive cells enhanced significantly. The maximum number of plaque forming cells was observed in group IV on the 6th day and a significant antibody titer (211.82) on the 15th day of treatment. Reduction in serum Gamma Glutamyl Transpeptidase (GGT), Glutathione (GSH) and nitric oxide (NO) was noticed. The above results indicate the presence of bioactive compounds in ascidians.

Keywords: *Phallusia nigra*, antitumor, immunomodulatory, EAC.

Introduction

Cancer is considered as one of the serious disease which is characterized by abnormal or uncontrolled growth. It has become a major cause of death and there is an urgent need for its control. The most important drawback of the current cancer therapeutic practices such as chemotherapy and radiation therapy is the suppression of immune system[1]. Plants have been a prime source of natural products for the treatment of various diseases and they are highly effective conventional drugs for the treatment of many forms of cancer[2-4]. As plants are exploited for various reasons, marine organisms, especially those that are a nuisance to the environment like biofoulers can be screened for anticancer activity. Many marine sedentary organisms produce components with unique structural pattern, for their chemical defence which do not occur in terrestrial animals. One promising group of bioactive metabolites is ecteinascidins, biosynthesized by the colonial asicidan *Ecteinascidia turbinata* which has strong antitumor properties[5]. Sponges, bryozoans and tunicates are important source of new active principles for drug development[6]. Bistratene A is a polyether compound from the colonial ascidian *Lissoclinum bistratum* and is one of the most cytotoxic compounds[7]. Cytotoxicity of ascidians to various cell lines have been reported worldwide. In India anticancer activity of the ascidian *Polyclinum indicum* against cervical cancer cells (HeLa) mediated through apoptosis induction has been reported[8]. But antitumor and immunomodulatory studies on ascidians of Indian waters especially *Phallusia nigra* is lacking. Hence a preliminary attempt has been made.

Material and Methods

Specimen collection and identification: Samples of *Phallusia nigra* were collected from the under surface of the barges of Tuticorin harbour. Identification up to the species level was carried out based on the key to identification of Indian ascidians[9].

Systematic position: Phylum: Chordata, Subphylum: Urochordata, Class: Ascidiacea, Order: Enterogona, Suborder: Phlebobranchia, Family: Ascidiidae, Genus: *Phallusia*, Species: *nigra*

Experimental animals: Adult swiss albino mice weighing 20-25 g were obtained from the breeding section, Central Animal House, Dr. Raja Muthiah Medical College, Annamalai University, Chidambaram, Tamilnadu. The animals were kept in air-controlled room, fed with normal mice chow and water ad libitum. The experiments were conducted according to the rules and regulations of Animal Ethical Committee, Government of India.

Preparation of powder and extract: The animal was dried at 45°C and powdered. Ten grams of the powder was soaked overnight in 100 ml of 70 percent ethanol and filtered. The filtrate was centrifuged at 10,000 rpm at 4°C for 10 minutes. The supernatant was collected and evaporated to get a residue, which was used for in vitro studies. For in vivo animal experiments it was resuspended in 1% gum acacia blended with

vanillin and administered intraperitoneally at different concentrations.

In vitro cytotoxic activity: EAC cells ($1X10^6$ cells) were incubated with various concentrations (0.05, 0.10, 0.20 and 0.40 mg/ml) of extract in a final volume of 1ml for 3hr at 37°C. The viability of the cells was confirmed by trypan blue dye exclusion method after incubation[10].

Experimental protocol: Healthy adult swiss albino mice were weighed and divided into five groups of six each. Group I acted as control, Group II, III and IV received 50, 100 and 150 mg/kg of extract and Group V was administered with standard drug Vincristin (0.08 mg/kg body weight). EAC cells ($2X10^6$ cells/mouse) were injected intraperitoneally for 9 days. On the 10^{th} day, body weight of the animal was noted. 24 hours after the last dose of the drug, one set of the animals were sacrificed and the weight of the vital organs such as spleen, thymus, liver, kidney and lungs were recorded and expressed as relative organ weights. Blood was collected from caudal vein and parameters such as Hb, total RBC, WBC and differential count were recorded 30 days after the administration of the extract. Serum GGT, cellular GSH and NO were estimated at different time points (5^{th}, 10^{th} and 15^{th} day) using standard procedure.

Solid tumor volume: Tumor was induced by injecting EAC cells ($2X10^6$ cells/animal) subcutaneously to the right hind limb of the animals for five groups. The radii of the tumor were measured using Vernier Calipers at 5 days intervals for one month starting with 15^{th} day. The volume of the tumor was calculated using the formula
$V=4/3\ \prod r1^2r^2$, where 'r1' and 'r' represent the major and minor diameter respectively[11]. This was compared with untreated control (Group I).

Median survival time and Percentage increase in life span (% ILS): The effect of animal extract was monitored by recording the mortality daily for six weeks and percentage increase in lifespan (% ILS) was calculated by the following equation.

$$\text{MST \%} = \frac{\text{Median survival time of treated group} - \text{Median survival time of control group} \times 100}{\text{Median survival time of control group}}$$

Median survival time (MST) =
(Day of first death + Day of last death)/2
Increase in lifespan = $\frac{T-C}{C} \times 100$

Packed cell volume: The mice were dissected and the ascitic fluid was collected from the peritoneal cavity. The volume was measured using a graduated centrifuge tube and packed cell volume determined by centrifuging at 10,000 rpm for 5 minutes.

Viable and non viable cell count: The cells were stained with trypan blue (0.4% in normal saline) dye. Those that did not take up the dye were viable and that which took the stain are non viable. The viable and non viable cells were counted.

Hematological parameters: Blood was collected from caudal vein of the experimental mice after thirty days and parameters such as haemoglobin (cyanmethemoglobin), RBC, WBC and differential count (Leishman's stain) was recorded.

Bone marrow cellularity and β esterase activity: Experimental animals treated for 5 consecutive days were sacrificed 24 hours after drug treatment. The bone cells were collected from the femur, made into single cell suspension and the number determined by using haemocytometer. Bone marrow cells from the above preparation was smeared on clear glass slide and stained with Harri's hematoxylin to determine the non-specific β esterase activity by the azodyle coupling method[12].

Circulating antibody titer: Swiss Albino mice were divided into five groups of 6 animals. Group I was immunized with SRBC (0.1ml, 20 %). Group II, III and IV were treated with different dose of extract and the last group with Vincristin along with 0.2 ml SRBC for 5 consecutive days. Blood was collected from caudal vein every 3^{rd} day after drug administration and continued for a period of 30 days. Serum was separated, heat-inactivated at 56°c for 30 minutes and used for the estimation of antibody titer using SRBC as antigen[13].

Antibody producing cells: To determine the effect of the extract on the antibody producing cells, half of the experimental animals from the above treatment were sacrificed on different days starting from the third day after immunization up to the 9^{th} day. Spleen was processed to single cell suspension and the number of plaque forming cells (PFC) was determined by the Jerne's plaque assay[14].

Serum Gamma Glutamyl Transpeptidase (GGT) and Nitric Oxide (NO) levels: Blood was collected from experimental animals at different time points (5^{th}, 10^{th}, 15^{th} day) and the serum was used for the estimation of GGT and NO levels[15,16].

Determination of cellular Glutathione (GSH) and Nitric Oxide (NO) levels: Blood was collected at different time points (5^{th}, 10^{th}, 15^{th} day) and the cells (1X10 cells/ml) were sonicated for 30 seconds and used for the estimation of GSH and NO[17,16].

Statistical Analysis: Values are expressed as mean ± SEM. The statistical analysis was done by one-way analysis of variance (ANOVA) followed by Dunnett's test. P-values less than 0.5 were considered to be significant.

Results and Discussion

Effect on invitro cytotoxic activity to EAC cells: The extract was found to be toxic at different concentration to EAC cells.

Concentration of 0.05, 0.10, 0.20 and 0.40 mg/ml showed 16, 45, 65, and 100 percentage cytotoxicity (figure-1).

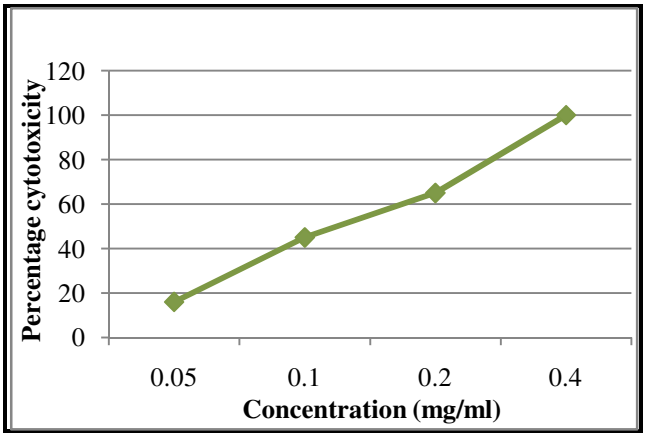

Figure-1
Invitro cytotoxic activity to EAC cells

Effect on organ weight: Table-1 shows the effect of the extract on organ weight. There was a significant decrease in the body weight of treated groups compared to the untreated control. A highly significant decrease was observed in group IV treated with 150 mg/kg of extract. The weight of vital organs such as spleen, liver and kidney increased where as that of thymus showed a dose dependent decrease. No significant change in lungs was noted. Thymus is a lymphoid organ which plays an important role in immunomodulations by activating the humoral and cellular immune system. The changes observed in the weight of the various organs of the treated group may be indicative of the stimulation of the production of immune related cells to fight against rapidly proliferating tumor cells. The liver is a major organ with macrophages, NK cells, NKT cells, which are key components of the innate immune system.

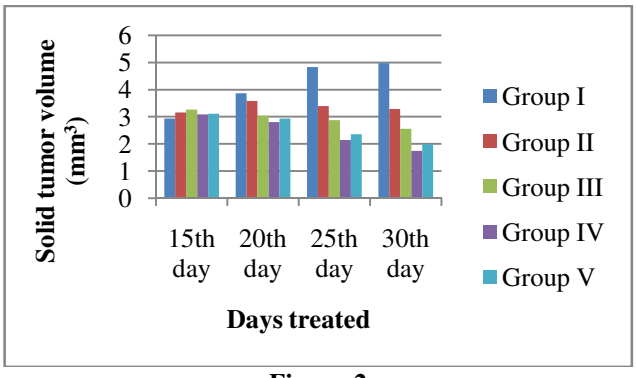

Figure-2
Effect of *Phallusia nigra* on solid tumor volume

Effect on tumor volume: A significant reduction in tumor volume was observed on 20th, 25th and 30th day in the treated mice compared to control (figure-2). The extract administration reduced tumor volume indicating inhibition in the growth and multiplication of tumor cells which may be due to the decrease in the ascites fluid acting as a direct nutritional source or the presence of compounds inhibiting mitosis, DNA synthesis or replication via enzyme pathways[18,19].

Effect on median survival time, lifespan, packed cell volume, viable and non viable cell count: Median survival time and percentage lifespan of mice increased in the treated groups. Life span increased by 68.42% when compared to normal control. An increase in the median survival time and life span indicates the activeness of the extract screened. Packed cell volume (2.36%) and viable cell count (3.83×10^6 cells/ml) decreased. Non-viable cell count (1.96×10^6 cells) increased significantly (table-2). This may be due to cytotoxic effect on tumor cells or by leading to macrophage activation and inhibition of vascular permeability by the extract[20].

Effect on hematological parameters: Haemoglobin and RBC content increased in treated groups. There was a significant reduction in the total WBC content in Group IV treated with 150 mg/ kg body weight of the extract. In the differential count, the lymphocytes increased where as neutrophils and eosinophils showed a decrease in treated mice (table-3) which indicates stimulation of the haemopoetic system. Suppression of the myeloid lineage and development of anaemia is a major problem observed during chemotherapy[21]. The anaemia encountered in tumor bearing mice is mainly due to reduction in RBC or hemoglobin percentage and this may occur either due to iron deficiency or due to hemolytic or myelopathic conditions[22]. Treatment with the extract of *Phallusis nigra* brought back the haemoglobin content, RBC and WBC cell count near to normal values. Low Neutrophil and Eosinophil content indicated that they started fighting off tumor progression.

Effect on Bone marrow cellularity and β Esterase activity: Extract treated animals showed significant increase in bone marrow cellularity ($24.85X10^6$ cells/femur) and β esterase positive cells (1267/4000 cells) (table-4). This observation indicates the presence of bioactive compounds bringing about an enhancement of the differentiation of stem cells and immunological response[23].

Effect on circulating antibody titer: The total antibody production increased significantly by the administration of the extract (table-5). The maximum antibody titer value of 211.82 was observed on 15th day in Group IV treated with 150 mg/ kg body weight. In the control animals the antibody titer value was only 178.66 on the same day. The stimulatory effect of the extract on the humoral arm of the immune system is indicated by an increase in the circulating antibody titer[24].

Effect on antibody forming cells: In group IV the maximum number of plaque forming cells (182.23 PFC /10^6 spleen cells) was observed where as in the control the number was 113.54 PFC/10^6 spleen cells on the sixth day (figure-3). A higher PFC may be due to an elevated antibody titer. The activation of

humoral immune response by the extract might have increased the plaque forming cells in spleen[23].

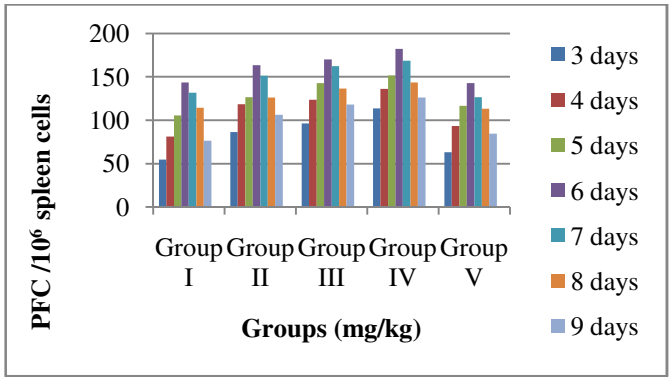

Figure-3
Effect of *Phallusia nigra* on antibody forming cells

Effect on serum GGT and NO levels: On the 15th day an enhanced level of GGT in the serum of control tumor bearing mice (112.46 nmol p-nitroaniline/ml) was observed. There was a significant reduction (41.65 nmol p-nitroaniline/ml) after the administration of the extract of *Phallusia nigra* (table-6). The intra and extra cellular glutathione concentration is maintained by GGT and it has been reported to play important role in antioxidant defence, detoxification and inflammation[25]. An oxidative stress during tumor induction might have increased the GGT level as an adaptive mechanism. A dose dependent decrease of GGT observed on the 15th day may be because of the transfer of Gamma Glutamyl Transpeptidase (GGT), from glutathione to amino acids and dipeptides[26]. The serum NO level was found to be maximum (39.63µM) on the 15th day of tumor progression in the control, which was reduced to 28.56 µM in group IV. Important physiological response and apoptosis is regulated by NO which is lipophilic, highly diffusible short lived messenger[27,28]. The reduction in NO may lead to an increase in cell death.

Effect on GSH and NO levels: The GSH content was maximum 14.63±0.86 nmol/mg protein on the 10th day of tumor growth in the control where as in the treated group the level decreased to 8.59± 0.48 and 6.05±0.37 on the 15th day (table-7). Production and metabolism of tumor cells requires GSH which is a major antioxidant involved in strengthening of immune system by producing T cells and changing the level of reactive oxygen species beneficial in reducing cancer growth[29]. In the control NO level increased gradually and was found to be highest (14.56±0.54) on 15th day of tumor progression. In group IV the NO level was restored to normal 5.14± 0.50. NO in tumor cells are supposed to exert anti cancer activity by altering the oxidation reduction status in tumor cells[30].

Table -1
Effect of *Phallusia nigra* extract on Relative Organ Weight of tumor induced mice

Group	Dose (mg/kg)	Relative Organ Weight (g/100g body weight)					
		Body weight	Spleen	Thymus	Liver	Kidney	Lungs
I	Control	36.59±2.08	0.47±0.018	0.28±0.014	3.51±0.19	2.11±0.015	0.69±0.056
II	50	27.53±1.39	0.53±0.31	0.19±0.034*	3.73±0.13	2.94±0.021	0.73±0.043
III	100	25.66±1.56*	0.74±0.011**	0.17±0.023*	3.95±0.24	3.24±0.039*	0.68±0.021
IV	150	21.14±1.08*	0.81±0.014***	0.14±0.016**	4.33±0.14*	3.91±0.026*	0.51±0.014*
V	Vincristin 0.08	29.54±1.91	0.53±0.23	0.24±0.24	4.04±0.33	3.11±0.019	0.65±0.026

Data represented as mean ±SEM, (N=6). Significance between EAC control and extract treated group. * $P < 0.05$, ** $P < 0.01$, *** $p < 0.001$

Table -2
Effect on median survival time, life span, packed cell volume, viable and non-viable cell count

Group	Dose (mg/kg)	Median Survival time (Days)	Increase of life span (%)	Packed Cell volume (%)	Viable cells 1X10⁶ cells/ml	Non-viable cells 1X10⁶cells/ml
I	Control	18.56±0.29	-	3.11±0.034	11.93±0.98	0.83±0.012
II	50	23.14±0.17	24.67	3.03±0.016	9.81±0.67	0.99±0.024
III	100	28.56±0.28*	53.75*	2.54±0.029	4.33±0.29**	1.74±0.056*
IV	150	31.26±0.51**	68.42**	2.36±0.014*	3.83±0.11***	1.96±0.039*
V	Vincristin 0.08	30.29±0.38*	63.20**	1.13±0.029*	2.29±0.32***	2.14±0.059**

Data represented as mean ±SEM, (N=6). Significance between EAC control and extract treated group. * $P < 0.05$, ** $P < 0.01$, *** $p < 0.001$

Table -3
Effect of the extract on hematological parameters in EAC Tumor bearing mice

Group	Dose (mg/kg)	Hb (gm %)	RBC (million/mm^3)	WBC (10^3 cells/ mm^3)	Differential Count (%)		
					Lymphocytes	Neutrophils	Eosinophils
I	Control	8.68±0.53	2.84±0.14	16.59±0.91	31.29± 0.78	50.17±1.97	18.54±1.15
II	50	10.84±1.08	3.36±0.23	12.14±0.74*	39.56±0.36	47.23±1.24	13.21±1.63
III	100	12.18±1.21*	3.98±0.54	11.33±0.29*	49.14±0.18*	40.26±1.72*	10.60±1.37*
IV	150	13.86±1.13**	4.36±0.22*	8.16±0.64**	57.33±0.27**	37.46±1.13**	05.07±1.01**
V	Vincristin 0.08	12.91±1.34*	4.21±0.13*	9.33±0.74**	62.16±0.13**	32.46±1.77**	05.38±1.17**

Data represented as mean ±SEM, (N=6). Significance between EAC control and extract treated group. * P < 0.05, ** P < 0.01

Table-4
Effect on Bone Marrow Cellularity and β-Esterase Activity in EAC Tumor bearing mice

Group	Dose (mg/kg)	Bone marrow cellularity (10^6 cells/femur)	β-Esterase activity (β-esterase positive cells /4000 cells)
I	Control	15.47±0.23	668±23
II	50	17.62±0.65	705±19
III	100	20.11±0.33*	1007±39*
IV	150	24.85±0.32**	1267±75**
V	Vincristin 0.08	20.67±0.51*	1141±73*

Each Value is ± SEM of 6 animals, Significance between tumor induced control vs drug treated group * P < 0.05, ** P < 0.01

Table-5
Effect on Antibody Titer in EAC Tumor bearing mice

Days Treated	Antibody titer				
	Group I	Group II	Group III	Group IV	Group V (Vincristin)
3	21.45±0.08	23.45±1.23	21.46±1.23	27.38±1.25	22.17±1.32
6	52.56±1.32	58.34±2.98	73.56±2.31**	79.36±2.11**	81.07±1.67**
9	87.24±1.98	93.22±2.78	128.48±2.92*	158.51±2.37**	162.39±2.13**
12	164.6±73.16	128.71±3.02	159.42±3.04	193.01±2.92**	201.48±3.56**
15	178.66±2.23	185.72±2.83	192.47±3.67	211.82±3.74*	234.68±3.64*
18	113.54±2.54	127.84±3.64	137.66±2.89	142.51±2.42*	146.50±2.92*
21	80.32±2.45	91.59±3.17	102.63±2.58	98.60±2.49	81.55±1.98
24	42.43±3.11	54.77±3.31	62.36±1.48	53.27±2.44	42.91±1.62
27	23.24±1.52	31.20±1.34	28.34±1.37	21.50±1.64	16.73±1.31
30	11.13±0.97	21.67±1.71	10.04±0.84	9.33±0.56	10.37±1.48

Each Value is ±SEM of 6 animals, Significance between tumor induced control vs drug treated group. * P < 0.05, ** P < 0.01

Table-6
Effect on the serum GGT and NO levels of EAC bearing animals

Group	Dose (mg/ml)	GGT (nmol p-nitroaniline/ml)			NO (µM)		
		5th day	10th day	15th day	5th day	10th day	15th day
I	T.Control	41.56±0.78	94.32±1.18	112.46±1.91	22.62±0.95	34.46±0.47	39.63±0.63
II	50	28.42±0.33*	64.59±0.68*	64.29±0.27*	19.69±0.45	29.80±0.34	36.34±0.63
III	100	24.64±0.84*	56.29±0.66*	58.57±0.38**	17.37±0.21*	25.48±0.52	32.47±0.49
IV	150	20.14±0.12**	48.36±0.58**	41.65±0.85***	14.56±0.32**	20.11±0.51*	28.56±0.63*
V	Vincristin 0.08	26.54±0.36*	40.16±0.34**	54.59±0.91**	16.39±0.21*	26.56±0.44	34.11±0.65

Each Value is ±SEM of 6 animals Significance between tumor induced control vs drug treated group * P < 0.05, ** P < 0.01, *** p<0.001

Table-7
Effect on the Cellular GSH and NO levels of EAC cells at different stages of Tumor growth in vivo

Group	Dose (mg/ml)	GSH (nmol/mg protein)			NO (µM)		
		5th day	10th day	15th day	5th day	10th day	15th day
I	Control	6.15±0.14	14.63±0.86	9.63±0.57	8.14±0.37	11.29±0.17	14.56±0.54
II	50	5.61±0.21	8.43±.33*	7.04±0.42	5.94±0.26	7.54±0.41	7.14±0.11
III	100	6.14±0.16	9.12±0.64	6.84±0.36*	5.13±0.67*	4.65±0.48*	6.84±0.15*
IV	150	7.26±0.73	8.59±0.48*	6.05±0.37*	5.03±0.46*	4.15±0.23*	5.14±0.50**
V	Vincristin 0.08	7.31±0.44	8.56±0.68*	6.71±0.61*	7.56±0.32	7.17±0.85	6.62±0.43*

Each Value is ±SEM of 6 animals Significance between tumor induced control vs drug treated group * $P < 0.05$, ** $P < 0.01$

Conclusion

A comparison of the results obtained for *Phallusia nigra* extract at a dose of 150 mg/kg body weight with that of standard drug indicates a significant antitumor and immunomodulatory activity. Further studies are needed to isolate the compounds responsible for the activity and pinpoint the mechanism involved. A preliminary GC-MS studies of the ethanolic extract has shown the presence of compounds like 2-Piperidinone, benzeneacetamide, tetradecanoic acid, n-hexadecanoic acid, 3-pentadecyl-phenol, (Z,Z,Z)- phenylmethyl ester of 6,9,12-octadecatrienoic acid, cholesterol, cholestan-3-ol, 3-hydroxy-, (3á,17á)- spiro[androst-5-ene-17,1'-cyclobutan]-2'-one and (Z)-phenylmethyl ester of 9-octadecenoic acid exhibiting anticancer, cancer preventive and antioxidant activity[31].

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