

IN VIVO ANTITUMOR ACTIVITY OF PHYTOCHEMICAL PITC-2 OBTAINED FROM TISSUE CULTURED PLANT *PLUCHEA INDICA* ON SARCOMA-180 SOLID TUMOR MICE MODELSOUMITA GOSWAMI¹, SOUVIK DEBNATH¹, SAUMEN KARAN¹, TAPAN KUMAR CHATTERJEE^{1,2*}¹Pharmacology Laboratory, Department of Pharmaceutical Technology, Jadavpur University, Kolkata - 700 032, West Bengal, India.²Department of Pharmacy, JIS University, Agarpara, Kolkata - 700 109, West Bengal, India.

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ABSTRACT

Objective: PITC-2 was isolated from the methanolic root extract of tissue cultured medicinal plant *Pluchea indica* (L.) Less. PITC-2 is a thiophene derivative which is 2-(Prop-1-ynyl)-5(5,6-dihydroxyhexa-1,3-dienyl)-thiophene. The main objective of the study is to evaluate the *in vivo* antitumor activity of PITC 2 against sarcoma-180 cancer cell in Swiss albino mice.

Methods: The antitumor activity was evaluated by treatment with PITC-2 at a dose of 2.5 and 5 mg/kg b.w for 21 days on sarcoma-180 mice model. Cell viability was studied using 3-(4, 5- dimethylthiazol -2-yl)-2, 5-diphenyl tetrazolium bromide assay and cell apoptosis, G₁ cell cycle arrest and reduction in tumor cell proliferation were evaluated by histopathological analysis and Bcl-2, cyclic-D1, and Ki-67 protein expression through immunohistochemistry study.

Results: Precisely, PITC-2 had a cytotoxic effect on various *in vitro* cancer cells. Significant decreases in solid tumor volume and weight along with increase lifespan also observed. The histopathological and immunohistopathological examination indicates that PITC-2 induces apoptosis, typical morphological changes and suppresses tumor cell proliferation along with G₁ cell cycle arrest through the downregulation of the intratumoral expression of Bcl-2, cyclic D1, and Ki-67 and thus highlighting antiproliferative and apoptotic properties against sarcoma-180 *in vivo* solid tumor model.

Conclusion: The present results clearly demonstrate that PITC-2 significantly inhibits sarcoma-180 cell growth in a dose-dependent manner in *in vivo* mice model. Besides this, the study reveals a comprehensive perception of the possible mechanism behind the antitumor activity of PITC-2 by significant changes in the morphological, hematological, biochemical parameters in sarcoma-180 cells.

Keywords: Apoptosis, Cell viability, PITC-2, Sarcoma-180, Antitumor activity, Cell proliferation.

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INTRODUCTION

Cancer and tumors are considered as the most killer disease all over the world. Cancer is caused by abnormalities of the genetic material of the affected cells. Tumorigenesis is a multistep process that involves the accumulation of successive mutations in oncogenes and suppressor genes that deregulates the cell cycle. It is a cellular disease in which the normal relationship between epithelial cells and their underlying stomach cells are altered [1]. Tumor metastasis is responsible for approximately 90% of all cancer-related death [2]. Although struggling efforts against cancer have grown tremendously in past few years, still it is the second leading cause of death in economically developed countries. An increase in worldwide death rate was found on cancer patients which are 7.1 million and 8.2 million on 2007 and 2012, respectively [3,4]. By 2030 the global burden is expected to grow to 21.7 million new cancer cases and 13 million cancer death [5].

Cancer therapy is based on surgery, radiotherapy, and systemic chemotherapy. Chemotherapy induces apoptosis which initiates tumor cell death. Apoptosis is an evolutionarily conserved form of death of a cell to maintain tissue homeostasis which allows removal of damaged or superfluous cells. This can be mediated either by death receptor pathway or by mitochondrial pathway. First indication of apoptosis is activation of several caspases within the same cell and second evidence from different timing of caspase activation after addition of apoptotic stimuli are consistent with a hierarchy of

caspase activation [6,7]. It is obvious that cancer chemotherapy is a very difficult task [8]. One of its main associated problems in the non-specific toxicity of most anticancer drugs due to their biodistribution throughout the body, which requires the administration of a large total dose to achieve high local concentrations in a tumor. Another problem in cancer chemotherapy is drug resistance. It may be due to cancer cell mutation or by repairing the DNA breaks caused by anticancer drugs or by adopting some other mechanism that inactivates the drugs. Numerous cancer research studies have been conducted using traditional medicinal plants in an effort to discover new therapeutic agents that lack the toxic side effects associated with current chemotherapeutic agents [9]. Recent interest in these secondary metabolites has been focused on their medicinal properties [10]. In this project, we are using a tissue cultured plant *Pluchea indica*. The plant was tissue cultured with the purpose to obtain more secondary metabolites [11]. *P. indica* (L.) Less. is a species of flowering plant in the family Asteraceae. The terminal flowers are rose purple and grow in rather loose few-flowered heads. It is an evergreen large shrub found abundantly in salt marshes and mangrove swamps in Sunderbans (India), Bangladesh, Myanmar, China, Philippines, Malaysia, Tropical Asia, and Australia [12]. From the methanolic root extract of tissue cultured medicinal plant, *P. indica* PITC 2 was isolated. It is a thiophene derivative which is 2-(Prop-1-ynyl)-5(5,6-dihydroxyhexa-1,3-dienyl)-thiophene. The main objective of the study was to evaluate the *in vivo* antitumor activity of PITC 2 against sarcoma-180 cancer cell in Swiss albino mice.

METHODS

Extraction and isolation

The roots after being separated from the whole plant were washed and dried at 60°C in a forced air oven. Dried roots are grounded and extracted separately in petroleum ether (60–80°C for synthesis) and then dried and extracted in a Soxhlet extractor with methanol (AR grade, purity 99.8%). After distillation of methanol, a sticky dark brown residue was obtained. For isolation of the phytochemical PITC 2 from tissue cultured plant *Pluchea indica*, the methanolic root extract was partitioned in a mixture of n-Butanol and water. From there butanol fraction was taken and then shaken with ethyl acetate to get an ethyl acetate soluble and insoluble part. Finally, ethyl acetate soluble part is taken and concentrated in a rotary vacuum evaporator and dried to obtain a crude residue which is then column chromatographed with a mixture of ethyl acetate and Petroleum ether. At a ratio 8:2 of pet ether and ethyl acetate from fraction 8–15 a prominent spot was observed in the thin-layer chromatography plate which is the desired phytochemical PITC-2 [2-(Prop-1-ynyl)-5(5,6-dihydroxyhexa-1,3-diynyl)-thiophene] (Fig. 1). The presence of light yellow solid PITC-2 was further confirmed by testing the isolated compounds on nuclear magnetic resonance spectroscopy and MASS spectroscopy [13].

Cell lines and cell culture

In vitro cancer cell lines were obtained from National Centre for Cell Science, Pune, India, and Sarcoma-180 cancer cells were collected from Chittaranjan National Cancer Research Institute, Kolkata, India. The *in vitro* cells were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% FBS at 37°C in CO₂ incubator in an atmosphere of humidified 5% CO₂ and 95% air. The cells were maintained by routine subculturing in tissue culture flasks. Tumor cell counts were done in a Neubauer hemocytometer using the Trypan blue dye exclusion method. Cell viability was always found to be 95% or more.

Cytotoxicity assay (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide [MTT] assay)

This test is based on MTT, which is reduced to a purple-blue soluble formazan by the living cells [14]. Experiments were performed in 96-well flat-bottomed culture plates (BD Biosciences, USA). MTT was dissolved in phosphate buffered saline (PBS) at 5 mg/ml. Different concentrations of PITC-2 (0.1, 0.5, 0.1, 5, 10, 20, 25, and 30 µg/ml) were added and the plate was incubated for 24 h. Following incubation with each compound for 24 h, 20 µL of MTT was added to each well. After incubation for 4 h at 37°C, the culture medium was removed, and the formazan crystals were dissolved in 200 µL dimethyl sulfoxide (DMSO) [15]. Absorbance (A) of formazan dye was measured at 570 nm using a microplate reader. The background absorbance was determined at 690 nm and subtracted from the 570 nm measurement [2].

The percentage of viable cells was determined by the following equation: [16]

$$\text{Viable cells (\%)} = \frac{\text{A of treated cells}}{\text{A of untreated cells}} \times 100$$

The cytotoxic activities of this compound PITC-2 were determined in human breast cancer cell MCF7, human lung cancer cell A549, human macrophage cell U937, and sarcoma S-180.

Animals and study groups

In vivo studies were carried out on female Swiss albino mice weighing 22±0.50 g of either. The animals were kept under standard conditions of 12:12 h light and dark cycle in polypropylene cages and fed with standard laboratory diet and water *ad libitum*. As the phytochemical PITC-2 was not freely soluble in water so 1%, DMSO solution was prepared to make it solubilize. All *in vivo* experiments were conducted as per the guideline of Animal Ethics Committee of the Department of the Pharmaceutical Technology of Jadavpur University, India (Registration number: 147/1999/CPCSEA).

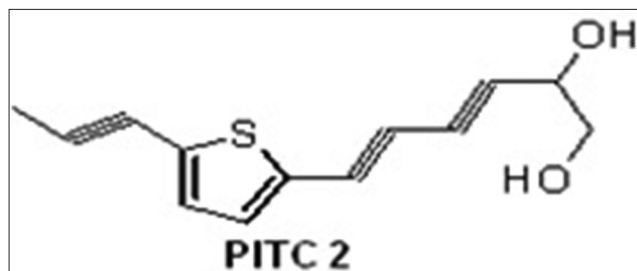


Fig. 1: 2-(Prop-1-ynyl)-5(5,6-dihydroxyhexa-1,3-diynyl)-thiophene

For experimental purpose, animals are divided into five groups each containing 12 mice.

1. Group I: Normal animals (negative control).
2. Group II: Sarcoma cancer animals (positive control)+1% DMSO 5 ml/kg/day.
3. Group III: Sarcoma cancer animals (test 1)+PITC 2 (2.5 mg/kg/day given orally).
4. Group IV: Sarcoma cancer animals (test 2)+PITC 2 (5 mg/kg/day given orally).
5. Group V: Sarcoma cancer animals (standard)+5FU (20 mg/kg in alternative day given i.p).

Solid tumor induction by sarcoma 180 (S-180) cell line and experimental protocol

Sarcoma-180 cells were preserved *in vivo* in the peritoneal cavity of mice by intraperitoneal inoculation of cells in sterile normal saline [17]. Then, solid tumors were inoculated in the left hind leg of mice by intramuscular injection of S-180 cells (0.2 ml of 2×10⁶ cells/mouse) to all the groups except the normal group [18]. Treatment was given from day 8 after inoculation of S-180 tumor for 21 days [19]. From each group, 6 mice were sacrificed, and other 6 were kept for calculation of mean survival time (MST).

Recording of survival rate

MST and percentage increase in lifespan (%ILS) were calculated by the following formulas [20].

$$\text{MST} = \frac{\sum \text{Survival time (day) of each mice in a group}}{\text{total number of mice}}$$

$$\% \text{ILS} = \left[\frac{\text{MST of treated mice}}{\text{MST of control mice}} \right] \times 100$$

Tumor volume

In case of solid tumor, tumor volumes were recorded using a vernier caliper and calculated as $V (\text{mm}^3) = (a^2 \times b) / 2$ where a (smaller diameter) and b (large diameter) are perpendicular [21]. Tumor growth inhibition ratio was calculated as follows: Inhibition ratio (%) = $[(A-B)/A] \times 100$ where A is average tumor weight in negative control and B is average in each treatment group [22]. However, in case of an ascitic tumor, peritoneal fluid was collected from the peritoneal cavity. Then, the volume was measured by taking it in a graduated centrifuge tube.

Hematological parameters

Hematological studies were performed by recording the hemoglobin content, red blood cell (RBC), and white blood cells (WBC) counts from freely flowing tail vein blood [23]. Differential WBC leukocyte counts were carried out including neutrophils, lymphocytes, and monocytes from Leishman stained blood smears of normal, sarcoma-180 control and PITC 2 treated groups, respectively.

Biochemical parameters

After 24 h of last dose and 18 h of fasting the blood sample were collected by puncturing retro-orbital plexus. Blood was used for the assay of biochemical

parameters serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), albumin, globulin, bilirubin, alkaline phosphatase (ALP), and serum protein to investigate liver function alteration and creatinine was measured as a renal function parameter.

Histopathology

The tumor, liver and kidney were excised and fixed in 10% buffered formalin and embedded in paraffin. +5- μ m sectioned was stained with hematoxylin and eosin (H and E) [24]. Histological analysis was performed under a light microscope (40 \times). The morphological changes were photographed and there compared.

Immunohistochemical analysis of Bcl-2, Ki-67, cyclin-D1

Tumor section was deparaffinized with xylene and dehydrated with ethanol. After blocking the slides with 5% bovine serum albumin in Tris-buffered saline, the sections were then stained with a primary antibody specific to Bcl-2, Ki-67, and cyclin-D1, at a concentration of 1 μ g/ml at 4°C overnight, followed by mouse monoclonal antibody. After washing sections with PBS, they were incubated with diaminobenzidine substrate and counterstained with Mayer's hematoxylin. Then, they were visualized in a digital camera installed on a light microscope [2,21].

Statistical analysis

For cytotoxicity studies, the confidence interval (IC₅₀) values and 95% CI were obtained by nonlinear regression. All *in vitro* studies were carried out in triplicate represented by independent biological evaluation. Data are extracted as a mean \pm standard error of the mean and were compared by one-way analysis of variance followed by Kruskal–Wallis test using SPSS statistical software of 20.0 version. $p < 0.05$ was considered to be statistically significant when compared with control.

RESULTS

In vitro cell viability assay

The phytochemical PITC 2 was screened for its cytotoxicity against human breast cancer cell MCF7, human lung cancer cell A549, human

macrophage cell U937, and sarcoma S-180 at different concentrations (0.1, 0.5, 01, 5, 10, 20, 25, and 30 μ g/ml) to determine the percentage of viable cells by MTT assay. MTT assay shows a significant effect on sarcoma S-180, MCF-7, A-549, and U-937 cancer cells (Fig. 2). This result indicates antiproliferative activity of PITC-2 in cancer cells. Among these cancer cells, PITC-2 is most potent for U-937 cells.

Inhibition of Sarcoma-180 solid tumor growth by PITC-2

PITC-2 at concentrations 2.5 mg/kg and 5 mg/kg shows a good reduction of solid tumor volume and weight at the end of 21 days compared to that of control group mice. PITC-2 treated groups also shows significant reduction of body weight (Fig. 3 and Table 1).

PITC-2 enhanced survival of sarcoma 180 bearing mice

As shown in Table 1 and Kaplan–Meier survival curve (Fig. 4a) a significant increase in survival time and lifespan of sarcoma-180 bearing mice treated with PITC-2 was found in comparison with sarcoma bearing control group. The %ILS was found to be dose-dependent (Fig. 4b).

Effect of PITC-2 on hematological parameters

An increase in hemoglobin level, RBC count, neutrophil level and decrease in WBC level, monocyte, and lymphocyte count was found in PITC 2 treated groups in a dose-dependent manner in compared to that of sarcoma-180 control group (Table 2).

Effect of PITC-2 on biochemical parameters

PITC-2 treated group's shows a significant decrease in SGPT, SGOT, and serum ALP parameters are found in a dose-dependent manner (2.5 and 5 mg/kg b.w) in comparison with sarcoma-180 control group. A dose-dependent increase in total protein was also found (Table 3).

Histopathology of liver and kidney tissue

Fig. 5a shows H and E stained section of liver of healthy mice which bears all the normal features, including circular hepatic portal vein and

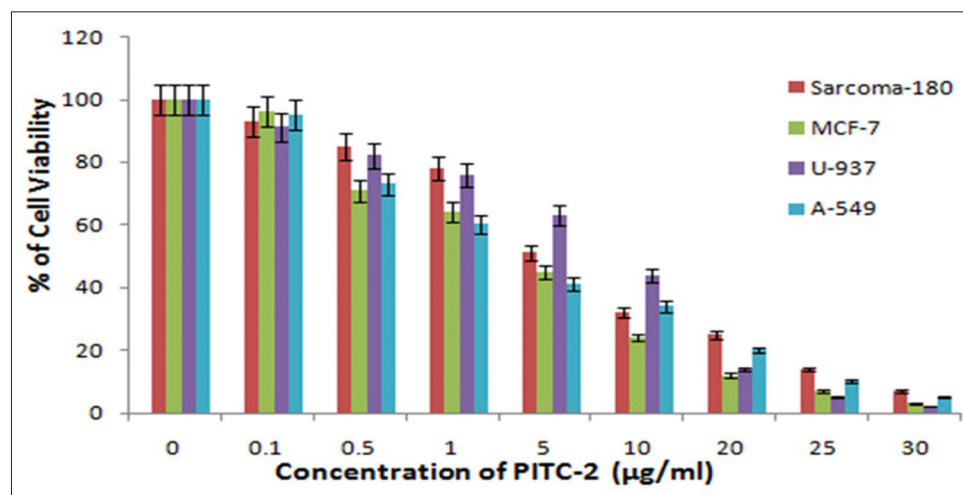


Fig. 2: *In vitro* cytotoxic activity of PITC-2 against Sarcoma 180, MCF-7, U-937, and A-549. Values are expressed as mean \pm standard deviation

Table 1: *In vivo* effect of PITC-2 on solid tumors and study groups

Parameters	PITC-2 2.5 mg/kg b.w	PITC-2 5 mg/kg b.w	Standard drug 5-FU 20 mg/kg b.w	Sarcoma-180 control
Tumor weight (g)	1.156 \pm 0.03*	0.649 \pm 0.008*	0.890 \pm 0.03*	3.90 \pm 0.11*
Tumor volume (mm ³)	1900.75 \pm 45.30	1563.10 \pm 27.65	1823.64 \pm 33.73	6642.30 \pm 51.34
Tumor inhibition ratio %	71.38 \pm 1.72*	76.46 \pm 0.27*	72.54 \pm 0.96*	0 \pm 0
Body weight (g)	24.05 \pm 0.43*	22.71 \pm 0.42*	23.68 \pm 0.55*	27.10 \pm 0.48*
MST (days)	34.67 \pm 0.98*	52.83 \pm 0.70*	42.00 \pm 0.85*	19.50 \pm 0.42*
%ILS	77.79	170.92	115.38	0 \pm 0

* $p < 0.05$ as compared to control group (n=6 mice per group). ILS: Increase in lifespan, MST: Mean survival time

Table 2: Effect of PITC-2 on hematological parameters in sarcoma-180 solid tumors bearing mice

Parameters	PITC-2 2.5 mg/kg b.w	PITC-2 5 mg/kg b.w	Std 5-FU 20 mg/kg b.w	Sarcoma-180 control	Normal mice
Hemoglobin (g/dl)	7.5±0.05*	10.4±0.03*	8.5±0.08*	6.3±0.01*	12.8±0.12*
Erythrocyte (RBC) (cells×10 ⁶ /mm ³)	4.34±0.04*	6.53±0.02*	5.6±0.03*	3.75±0.04*	7.90±0.02*
Leukocytes (WBC) (cells×10 ⁶ /mm ³)	14.1±0.05*	10.19±0.05*	4.29±0.05*	16.69±0.02*	6.40±0.03*
Neutrophil (%)	42.3±0.03*	39.46±0.03*	30.1±0.02*	68.5±0.05*	19.4±0.03*
Lymphocyte (%)	48.0±0.04*	51.0±0.04*	63.0±0.09*	35.8±0.11*	64.1±0.23*
Monocyte (%)	1.5±0.03*	1.4±0.03*	1.7±0.03*	1.3±0.04*	2.8±0.01*

*<0.05 as compared to control group (n=6 mice per group). RBC: Red blood cell, WBC: White blood cells

Table 3: Effect of PITC-2 on biochemical parameters in sarcoma-180 solid tumors bearing mice

Parameters	PITC-2 2.5 mg/kg b.w	PITC-2 2.5 mg/kg b.w	Standard 5-FU 20 mg/kg b.w	Sarcoma 180 control	Normal mice
Bilirubin (total) mg/dl	0.20±0.02*	0.26±0.02*	0.29±0.03*	0.34±0.01*	0.4±0.02*
Serum protein (total) g/dl	8.12±0.09*	9.69±0.07*	5.22±0.12*	15.4±0.08*	8.19±0.08*
AST (SGOT) U/L	0.71±0.01*	0.60±0.01*	0.59±0.01*	0.6±0.09*	0.8±0.01*
ALT (SGPT) U/L	351±0.04*	175±0.01*	178±0.06*	199±0.07*	414±0.04*
Serum ALP U/L	73±0.02*	68±0.02*	18±0.05*	40±0.03*	74±0.03*
Creatinine Mg/dl	1±0.01*	1±0.01*	1±0.01*	1±0.02*	1±0.01*

*<0.05 as compared to control group (n=6 mice per group). AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, ALP: Alkaline phosphatase, SGOT: Serum glutamate oxaloacetate transaminase, SGPT: Serum glutamate pyruvate transaminase

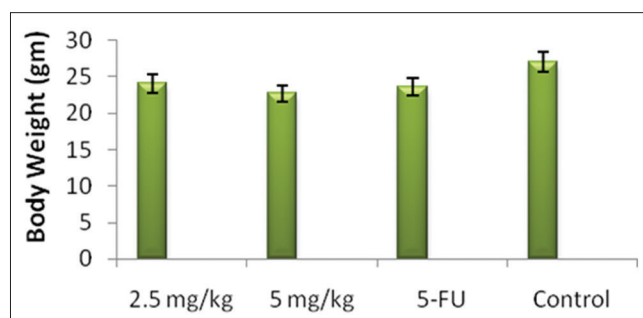


Fig. 3: Effect of PITC-2 treatment on body weight

of hepatic artery, as marked by arrow. It shows prominent nuclei, and the tissue section comprises hepatic sinusoid which is usual. However, in case of the Sarcoma-180 control group showed in Fig. 5b none of the above-mentioned regular features are observed, rather it shows extreme hepatocellular lesions as pyknotic nuclei, exhibiting necrotic hepatocytes. Group receiving standard 5-FU shows a lesser amount of hepatocellular lesion, which is close to normal as shown in Fig. 5c. Although little changes are found such as deformed hepatic artery and irregular bile duct. Groups receiving a different dose of PITC-2 also show a little hepatic deformation and altered hepatocyte population shown in Fig. 5d and e. Healthy hepatocytes were observed, regular branch of bile duct found, although mild dilation of central vein observed.

Fig. 6a shows H and E stained section of kidney of a healthy mice which bears all the normal features, while sarcoma-180 control group showed in Fig. 6b show severe glomerular and tubular hemorrhage with increase swelling of tubular epithelium and presence of hyaline cylinders. Group treated with 5-FU and PITC-2 shows cellular features which have less deformation and close to normal in Fig. 6c-e, respectively, with light glomerular and tubular hemorrhage.

Histopathological changes of solid sarcoma-180 tumor

Histopathological examination of solid sarcoma-180 tumor control group shows intact anaplastic cancer cells with areas of necrosis and focal hemorrhage reflecting viability and aggressiveness of tumor (Fig. 7a). It shows sheets of large rounded and polygonal cells with hyperchromatic nuclear and binucleation. Whereas solid tumor of group treated with 5-FU (Fig. 7b) and PITC-2 with different doses

(2.5 mg/kg and 5 mg/kg) (Fig. 7c and d) shows areas of necrosis. Low cell proliferation and muscle invasion were found, and with an increase in dose, tumor cells lose their details and architecture.

Immunohistochemical analysis of Bcl-2, Ki-67, and cyclin-D1

The tumor cells were considered positive Bcl-2, Ki-67, and cyclin-D1 with the presence of yellow-brown nuclear staining in Ki-67 (Fig. 8) and greenish brown nuclear staining in Bcl-2 (Fig. 9) and cyclin-D1 (Fig. 10). Here we are studying the regression in tumor growth by PITC-2, due to apoptosis that decreases expression of antiapoptotic protein Bcl-2, G1 cell cycle arrest by suppression of cyclin-D1 cell cycle protein. Furthermore, PITC-2 reduces tumor cell proliferation by decreasing Ki-67 tumor proliferation protein. A significant decrease in positive Bcl-2, Ki-67, and cyclin-D1 were found in PITC-2 treated group, compared to that of sarcoma-180 control group was observed which shows effective suppression of tumor. Meanwhile, it was found that the decrease in protein expression is dose-dependent and is better in case of PITC-2 with dose 5 mg/kg b.w.

DISCUSSION

Cancer is a group of disease which involves abnormal growth of cell which is rapid and uncontrolled with the potential to spread in other parts of the body. Anticancer drugs or antineoplastic drugs are drugs effective in the treatment of a malignant disease which trigger signaling mechanism that initiates apoptosis or cell death [25]. At present, available synthetic drugs have many adverse reactions which can be minimized to a great extent by the natural compound. The aim of the study was to evaluate the *in vivo* antitumor activity of PITC-2 against sarcoma-180 cancer cell in Swiss albino mice. Therefore, we established a mouse model bearing sarcoma-180 to study the antitumor activity of the drug. The mice treated with PITC-2 at different dose inhibit body weight, tumor weight, tumor volume, tumor inhibition ratio and also brought hematological parameters to more or less normal level which is dose-dependent. A same type of finding was observed by de Costa *et al.* on *in vivo* anticancer and antiangiogenic potential of thalidomide derivatives [22]. This study also shows inhibition of body weight, tumor volume, and tumor inhibition ratio. A good ILS and survival time was also noted in sarcoma-180 solid tumor-bearing mice when treated with PITC-2. A major problem in cancer chemotherapy is myelosuppression and anemia. Tumor-bearing mice have anemia mostly due to a reduction in RBC or low hemoglobin percentage. This may occur due to iron deficiency or due to myelopathy condition [23]. Treatment with

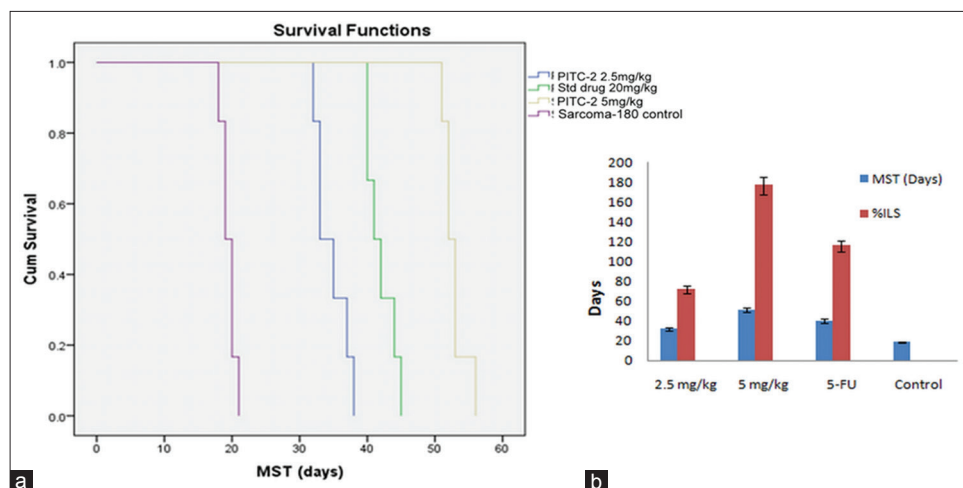


Fig. 4: (a) Kaplan–Meier survival curve showing survival of all groups, (b) effect of PITC-2 treatment on mean survival time, and percentage of increase lifespan

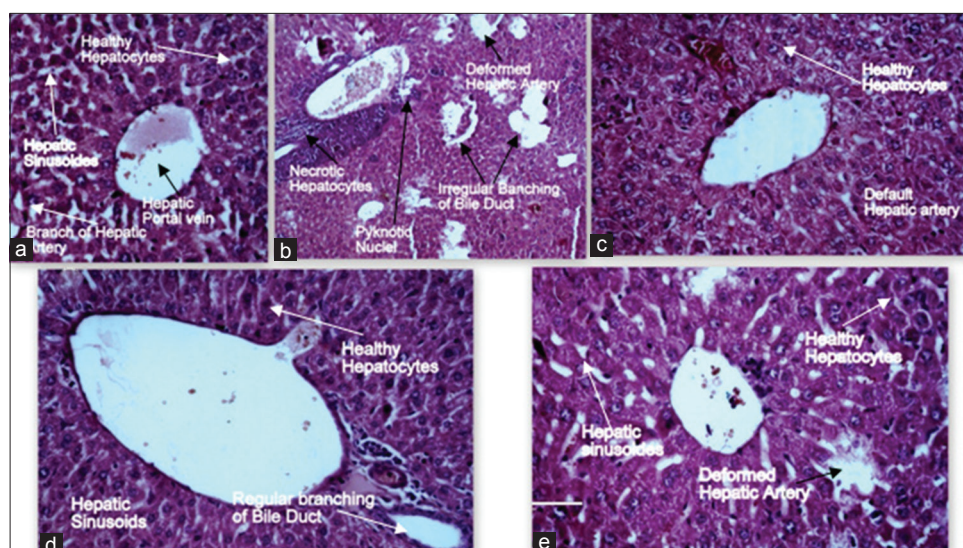


Fig. 5: Hematoxylin and eosin stained section of mice liver showing cellular changes for (a) normal healthy mice, (b) sarcoma-180 control group, (c) standard 5-FU treated group, (d) 2.5 mg/kg of PITC-2, and (e) 5 mg/kg of PITC-2 (magnification 40×)

PITC-2 stabilizes hemoglobin concentration, RBC and WBC cell count near to normal value. This reveals the protective activity of PITC-2 on the hematopoietic system. Another problem of cancer chemotherapy is liver toxicity which is due to accumulation of drug metabolites in liver [26]. In this study, liver toxicity was analyzed by biochemical studies and histopathology of liver tissue. Aspartate aminotransferase (AST), alanine aminotransferase (ALT), and ALP levels of control group mice, normal mice, and drug-treated mice were compared, and it was found that PITC-2 treated mice had less toxic liver than others as the level of AST, ALT, and ALP are close to normal value. These all features such as decrease in body weight, tumor weight, tumor volume, ILS and survival time, stabilizing hematological, and biochemical parameters of PITC-2 are dose-dependent, that in stabilizing effects are better in animals treated with higher dose of PITC-2. These features help in judging the drug activity.

Histopathological analysis of kidney for control, 5-FU, and PITC-2 for different dose was done. It was found that several degrees of hydropic changes of proximal tubular epithelium, glomerular, and tubular hemorrhage. These findings were much more prominent in control group animal than drug-treated groups where these effects are low, but the glomeruli structures were essentially preserved. The

necrosis is characterized by no nuclear staining and deeply eosinophilic cytoplasm [27]. This show PITC-2 is less toxic to kidney than standard drug. Many drugs can cause lesion of liver found on biopsy. A large number of drugs with different pharmacological action and chemical structure give rise to liver lesion. Although liver shows a great adaptive and regenerative ability, for example, if anticonvulsant drugs are used for a long time, it causes an increase in endoplasmic reticulum which is regarded as an adaptive phenomenon. Again regeneration of hepatic tissue occurs by the conjugative tissues after hepatocellular necrosis due to treatment with alginates [27]. In our study, normal healthy mice show prominent nuclei and the tissue section comprises hepatic sinusoid which is usual. However, in case of sarcoma-180 control group none of the above mentioned regular features are observed, rather it shows extreme hepatocellular lesions as pyknotic nuclei, exhibiting necrotic hepatocytes. Group receiving a different dose of PITC-2 are close to normal although little changes are found such as deformed hepatic artery and irregular bile duct which show PITC-2 is less toxic to liver.

From MTT assay it was found that PITC-2 has notable *in vitro* cytotoxic effect on S-180, MCF-7, A-549, and U-937 cancer cells. The mechanism of apoptosis is its diverse alteration of cells. Hence, cell apoptosis

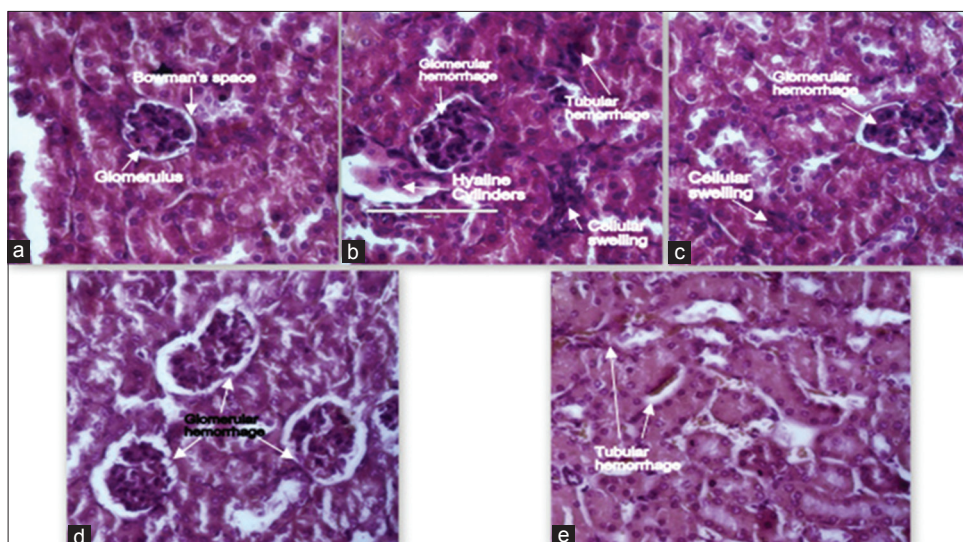


Fig. 6: Hematoxylin and eosin stained section of mice kidney for (a) normal healthy mice, (b) sarcoma-180 control group, (c) standard 5-FU treated group, (d) 2.5 mg/kg of PITC-2, and (e) 5 mg/kg of PITC-2 (magnification 40 \times)

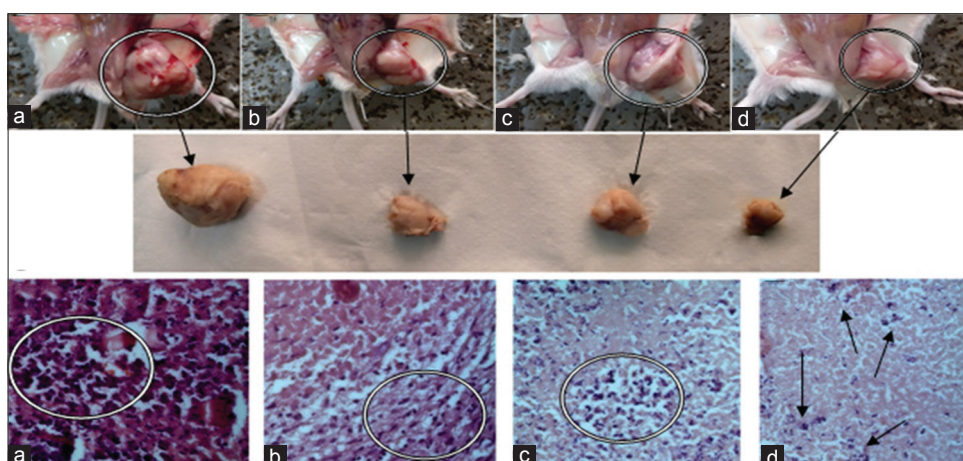


Fig. 7: Effect of PITC-2 treatment on solid sarcoma-180 tumor: (a) Sarcoma-180 control group, (b) standard 5-FU treated group, (c) 2.5 mg/kg of PITC-2, (d) 5 mg/kg of PITC-2, panel (1) shows tumor growth on animals, panel (2) shows tumor isolated from animal body, panel (3) hematoxylin and eosin stained histological section of solid tumor (magnification 40 \times). The cellular features are marked by circle

is identified by antiapoptotic protein Bcl-2 [28,29]. Bcl-2 plays an important role in apoptosis regulation in physiological and pathological conditions. Prevention of the loss of mitochondrial membrane potential is one of its notable works. It also prevents the release of cytochrome c into the cytosol which activates caspases by the mitochondrial pathway. Overexpression of Bcl-2 inhibits caspase-3 activation [30,26]. In our study, it was found that after treatment with PITC-2 on sarcoma-180 cells a significant decrease in Bcl-2 was observed whereas an increase in level Bcl-2 was observed in control group animals. This result concludes that increase of Bcl-2 would be a cellular response to counteract the apoptosis which is already initiated. Therefore, PITC-2 reduces the ability of Bcl-2 expression and initiates apoptosis.

To identify the tumor proliferation rate the cells, tumor cells are stained with Ki67. Nuclear staining was observed in all tumor sections which are immunized with Ki67 antibody. Mouse monoclonal antibody of Ki67 was used that identifies a nuclear antigen associated with G1, S, G2, and M phase. The expression of this molecule is found in all cell cycle except in G0 and early G1 phase [31]. In our study, it was found that a decrease in positive Ki67 cells in animals treated with 5FU and PITC-2 when compared to control group. It was also noted that reduction in tumor

proliferation rate is more for a higher dose of PITC-2.

Cyclic D1 overexpression is an indication of early cancer onset and tumor progression [32]. Cyclic D1 is a protein required for progression through G1 phase during the cell cycle. It synthesizes rapidly during G1 phase and accumulates in the nucleus. Further, it starts degrading when it enters S phase [33]. Our study shows a marked decrease in expression of Cyclic D1 in animals treated with 5FU and PITC-2 when compared to control group. Even more effective result was shown by a different dose of PITC-2 than 5FU which reveals antitumor activity PITC-2.

CONCLUSION

The present results clearly demonstrate that PITC-2 significantly inhibits sarcoma-180 cell growth in a dose-dependent manner in *in vivo* mice model. Besides this, the study reveals a comprehensive perception of the possible mechanism behind the antitumor activity of PITC-2 by significant changes in the morphological, hematological, and biochemical parameters in sarcoma-180 cells. Moreover, the histopathological and immunohistopathological examination indicates that PITC-2 induces apoptosis and suppresses tumor cell proliferation along with G1 cell

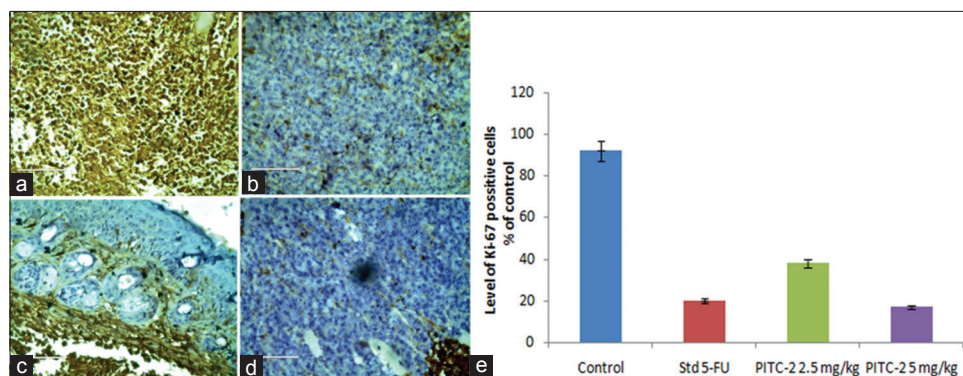


Fig. 8: (a) Sarcoma-180 control, (b) standard 5-FU, (c) PITC-2 2.5 mg/kg, and (d) PITC-2 5 mg/kg shows Ki67 expression (magnification 40 \times). (e) Shows level of immunopositive Ki67 cells, values are given in mean \pm standard deviation

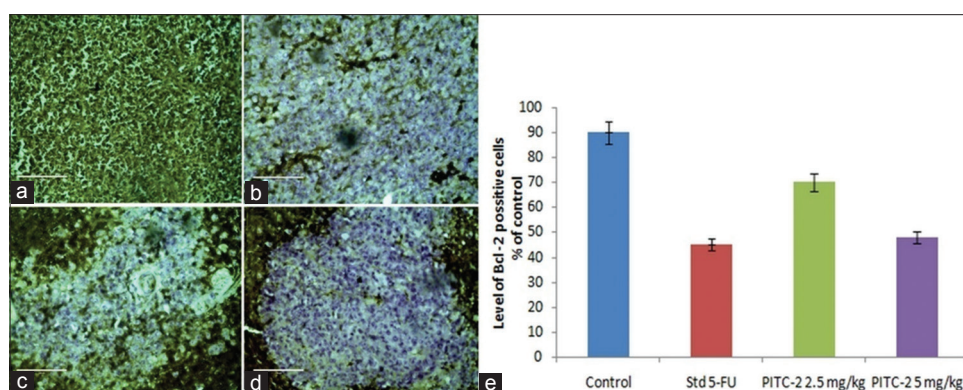


Fig. 9: (a) Sarcoma-180 control, (b) standard 5-FU, (c) PITC-2 2.5 mg/kg, and (d) PITC-2 5 mg/kg shows Bcl-2 expression (magnification 40 \times). (e) Shows level of immunopositive Bcl-2 cells, values are given in mean \pm standard deviation

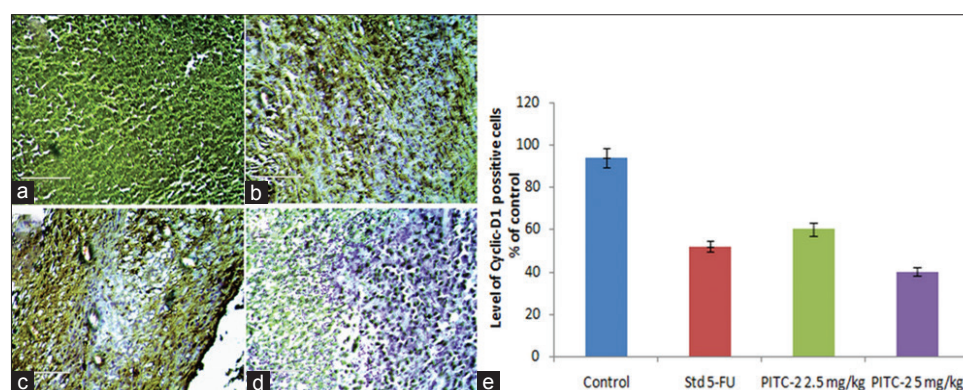


Fig. 10: (a) Sarcoma-180 control, (b) standard 5-FU, (c) PITC-2 2.5 mg/kg, and (d) PITC-2 5 mg/kg shows cyclic-D1 expression (magnification 40 \times). (e) Shows level of immunopositive cyclic-D1 cells, values are given in mean \pm standard deviation

cycle arrest through the downregulation of the intratumoral expression of Bcl-2, cyclic D1, and Ki-67 and thus highlighting antiproliferative and apoptotic properties against sarcoma-180 solid tumor model. Along with this, it shows no significant manifestation of toxic symptoms on liver and kidney of Swiss albino mice. Taking these results, we suggest that PITC-2 may be a potent prognostic factor and a potential target for tumor cells. Further investigations are in progress in our laboratory to identify the active principal and mechanisms involved in antitumor activity and to prepare a suitable formulation for modifying its way of administration.

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AUTHORS CONTRIBUTION

Authors make substantial contributions in design, analysis and interpretation of data. Authors also participate in drafting the article. Authors give final approval of the version to be submitted and revised version

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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