

In vivo Antineoplastic Activities of Benzoin Thiosemicarbazone and Para-Anisaldehyde Semicarbazone against Ehrlich Ascites Carcinoma Cells

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ABSTRACT

Two Schiff bases, namely benzoin thiosemicarbazone (BTSC) and para-anisaldehyde semicarbazone (PAS), were synthesized and characterized. The antineoplastic activities of these Schiff bases were studied against Ehrlich ascites carcinoma (EAC) cells in Swiss albino mice by monitoring tumor cell growth inhibition, tumor weight measurement, survival time of tumor-bearing mice, bioassay of these compounds, and counting the total number of peritoneal cells as well as macrophages. Hematological parameters were also studied. It was found that these compounds significantly reduced tumor cell growth rate, decreased tumor weight, and increased life span of EAC-bearing mice. These test compounds restored the hematological parameters of EAC-bearing mice to normal. These compounds also enhanced the number of macrophages in normal mice. The results obtained were compared with those obtained with the standard drug bleomycin.

Key words: Antineoplastic activity, benzoin thiosemicarbazone, EAC cells, para-anisaldehyde semicarbazone

INTRODUCTION

Cancer, which is a diverse group of diseases characterized by proliferation and spread of abnormal cells, is a major worldwide concern. Therefore, the discovery and development of new potent and selective anticancer drugs are of high importance in modern cancer research. Schiff bases containing the C=N group play a very important role in many biological activities. They have been found to possess anticancer (1,2), antimicrobial (3), antitubercular (4), anti-inflammatory and analgesic (5), antiviral (6), and pesticidal (7) properties. Not only the Schiff bases, but also Schiff base complexes with transition metals have been investigated for their anticancer activities against Ehrlich ascites carcinoma (EAC) cells in Swiss albino mice (8,9). Recently, the anticancer activities of some semicarbazones and thiosemicarbazones against EAC cells have been reported (10-12). The aim of the present study was to explore the anticancer properties of two Schiff bases, namely benzoin thiosemicarbazone (BTSC) and para-anisaldehyde semicarbazone (PAS).

MATERIALS AND METHODS

Chemicals

All chemicals and reagents used to carry out the research work were of reagent grade.

Experimental animals

Swiss albino mice, 5–7 weeks old and weighing 25–30 g, were collected from International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR'B) Mohakhali, Dhaka.

Animal care

Mice were kept in iron cages with saw dust and straw bedding, which was changed once a week regularly. Standard mouse diet (recommended and prepared by ICDDR'B) and water were given adequately.

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TABLE 1: Physical constants and IR spectral data of BTSC and PAS.

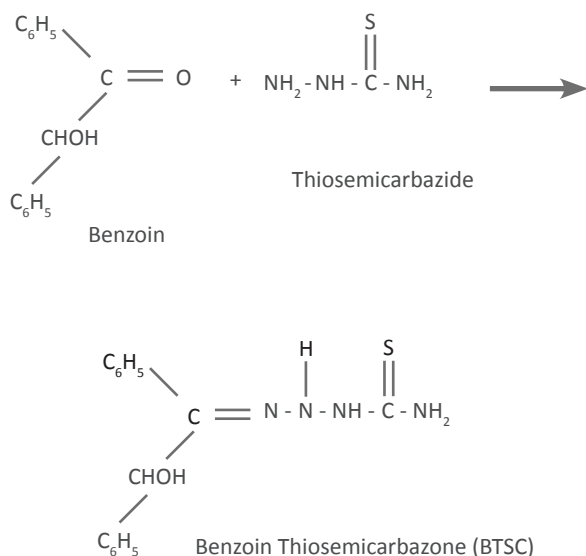
Test compounds	Yield (%)	Melting point (°C)	Physical form	Solubility	IR Spectra (cm ⁻¹)
BTSC	60	150–153 ¹⁵	White Crystalline	Ethanol DMSO	3164 (-NH-), 1284 (-C=N-NH), 940 (-C=S), 1607 (>C=N-)
PAS	65	204–207 ¹⁴	White Crystalline	Ethanol, DMSO	1650 (>C=N), 1690 (>C=O), 3500 (-CONH ₂)

Ethical clearance

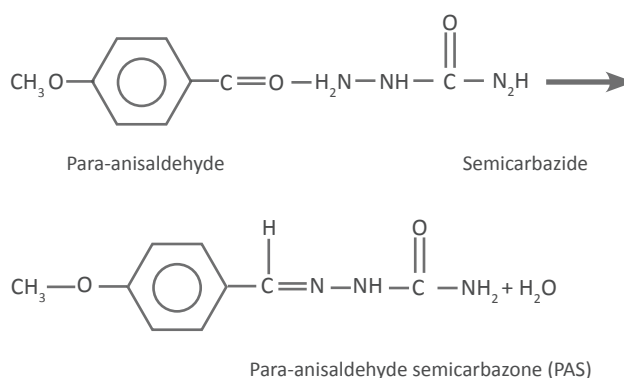
The protocol used in this study for the use of mice as the animal model for research was approved by the University Animal Ethical Committee (27/08/RUBCMB).

Synthesis of the compounds

These compounds were synthesized according to the method described in the literature (10–14). For preparing BTSC, benzoin and thiosemicarbazide were mixed in 1:1 molar ratio, refluxed for a period of 3–4 h, and then distilled to half of the total volume. The solution was then allowed to stand overnight till a white crystalline product separated out. The crystals were washed with ethanol, recrystallized, finally dried in an oven at 50°C, and stored in a desiccator.



For preparing PAS, para-anisaldehyde and semicarbazide (1:1 molar ratio) were mixed together and the same procedure was followed as used for BTSC.



Characterization of Schiff bases

The synthesized compounds were characterized by evaluating the melting point and IR spectra (on KBr disk using a Shimadzu FTIR) (Table 1).

Cell lines

Ehrlich ascites carcinoma (EAC) cells were obtained by the courtesy of Indian Institute of Chemical Biology (IICB), Kolkata, India. The cells were maintained as ascites tumor in Swiss albino mice by intraperitoneal inoculation (biweekly) of 2×10^6 cells/mouse.

Toxicity study

An acute toxicity study related to the determination of LD₅₀ was performed using the conventional method (16). The compounds were dissolved in 2% dimethyl sulfoxide (DMSO) and injected intraperitoneally into six groups of mice (each containing six mice) in different doses. LD₅₀ values were evaluated by recording mortality after 24 h.

Cell growth inhibition

In vivo tumor cell growth inhibition was carried out using the method as described (10–12) earlier. For this study, eight groups of mice (six in each group) were used. All the mice were injected with EAC cells (0.1 mL of 2×10^6 cells/mouse) intraperitoneally.

Treatment was started after 24 h of tumor inoculation and continued for 6 days. Groups 1–3 were treated with BTSC at the doses of 4 mg/kg, 8 mg/kg, and 16 mg/kg (i.p.), respectively. Groups 4–6 were treated with PAS at the doses of 10 mg/kg, 20 mg/kg, and 30 mg/kg (i.p.), respectively. Group 7 received standard drug bleomycin (0.3 mg/kg, i.p.). Group 8, treated with only normal saline (0.98%), was considered as untreated control. The mice of all the groups were sacrificed on the 6th day after transplantation, and tumor cells were collected by repeated intraperitoneal wash with 0.98% saline. Viable tumor cells per mouse of the treated groups were compared with those of control. The cell growth inhibition was calculated using the following formula:

% Cell growth inhibition = $(1 - I_w / C_w) \times 100$, where

I_w = mean of number of tumor cells of the treated group of mice

C_w = mean of number of tumor cells of the control group of mice.

Average tumor weight and survival time

The antitumor activities of BTSC and PAS were assessed (17) by measuring the average tumor weight, mean survival time (MST), and percentage increase in life span (%ILS). The treatment was continued for 10 days. Tumor growth was monitored daily by measuring the change in weight. MST of each group was monitored by recording the survival time. MST and %ILS were calculated using the following equations.

MST = Survival time (days) of each mouse in a group / Total number of mice

Percent increase in life span,

%ILS = (MST of treated group / MST of control group) \times 100

Bioassay of EAC cells

The procedure was a modification of the methods used in the literature (18). Three groups of mice (four in each) were inoculated with 2×10^6 EAC cells. Group 1 was treated with BTSC at the dose of 16 mg/kg (i.p.), and Group 2 was treated at the dose of 30 mg/kg (i.p.), for five consecutive days. Group 3 served as control. On day 6, mice of all the groups were sacrificed and tumor cells from each group were harvested in cold saline (0.98%), pooled, and

centrifuged. These cells were re-inoculated (2×10^6 cells/mouse i.p.) into three fresh groups of mice ($n = 4$) as earlier. No further treatment was given to these mice. On day 5, mice from each group were sacrificed and tumor cells per mouse were counted and compared with that of control.

Hematological studies

The hematological parameters, namely WBC, RBC, and hemoglobin contents, were determined by the standard methods (19) using cell dilution fluids and hemocytometer. Blood was collected from the mice by tail puncture. Nine groups of mice ($n = 4$) were used for this test. Groups 1–3 were treated with BTSC at the doses of 4 mg/kg, 8 mg/kg, and 16 mg/kg (i.p.), and groups 4–6 were treated with PAS at the doses of 10 mg/kg, 20 mg/kg, and 30 mg/kg (i.p.), respectively. Group 7 was used as control, Group 8 comprised normal mice, and Group 9 was treated with standard drug bleomycin 0.3 mg/kg (i.p.). Treatment started after 24 h of tumor transplantation and was continued for 10 consecutive days. On days 5, 10, 15, and 25, the blood parameters were assayed for all the groups.

Determination of the effect of Schiff bases on normal peritoneal cells

The effects of Schiff bases on normal peritoneal cells were determined (20) by counting total peritoneal cells and macrophages. Two groups of mice (four in each) were treated with BTSC and PAS separately at the dose of 16 mg/kg and 30 mg/kg (i.p.), respectively, for three consecutive days. The untreated group was used as control. After 24 hours of the last treatment, each animal were injected with 5 mL of normal saline (0.98%) into the peritoneal cavity and then sacrificed. Intraperitoneal exuded cells and macrophages were counted with 1% neutral red using a hemocytometer.

Statistical analysis

The experimental results were expressed as the mean \pm SEM. Data were calculated using one-way analysis of variance followed by Dunnett t test using SPSS software version 10 (An IBM Company, IBM Corporation, 1 New Orchard Road, Armonk, New York 10504, 1722, United States).

TABLE 2: Effect of the Schiff bases and bleomycin on cell growth inhibition

Experiment	Dose (mg/kg, i.p.)	No. of EAC cells in mice on day 6 after tumor cell inoculation ($\times 10^7$)	% Cell growth inhibition
Control (untreated EAC cell-bearing mice)	-	2.517 \pm 0.182	-
EAC + Bleomycin	0.3	0.297 \pm 0.012***	88.2
EAC + BTSC	4	0.99 \pm 0.014***	60.61
	8	0.66 \pm 0.02***	73.53
	16	0.32 \pm 0.003***	87.24
EAC + PAS	10	2.324 \pm 0.05**	35.77
	20	1.426 \pm 0.043***	60.58
	30	0.857 \pm 0.075***	76.29

Mice were inoculated with 2×10^6 EAC cells/mouse (i.p.) on day 0. Treatment was started after 24 h of tumor cell transplantation. The number of mice in each experiment was six ($n = 6$); the results were expressed as mean \pm SEM (standard error of mean). Treatment was continued for six consecutive days. Significance values were * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ when compared with the control.

RESULTS

The toxicity of these compounds was evaluated by measuring LD_{50} values. For BTSC and PAS, the values were found to be 75 and 220 mg/kg (i.p.), respectively.

In vivo tumor cell growth was observed with BTSC at doses 4 mg/kg, 8 mg/kg, and 16 mg/kg (i.p.) Maximum cell growth inhibition (87.24%) was found after treatment with BTSC at the dose of 16 mg/kg (i.p.). Treatment with PAS the dose of 30 mg/

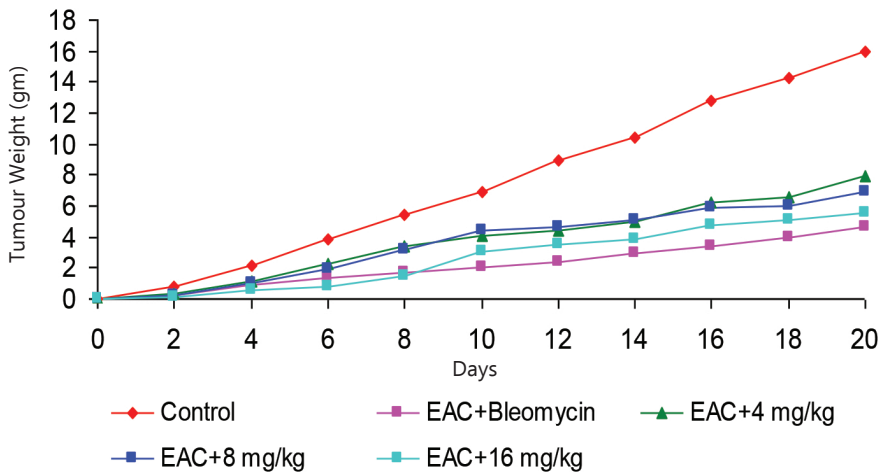
kg (i.p.) resulted in cell growth inhibition by 76.29%. On the other hand, bleomycin at the dose of 0.3 mg/kg (i.p.) inhibited the cell growth by 88.2% (Table 2).

The mean survival time (MST) of the untreated tumor-bearing mice was 23 days. With the treatment of test compounds, this value increased. About 78.26% enhancement of life span was found with 16 mg/kg (i.p.) BTSC and 55.29% with 30 mg/kg (i.p.) PAS at (Table 3).

TABLE 3: Effect of the Schiff bases and bleomycin on cell growth inhibition

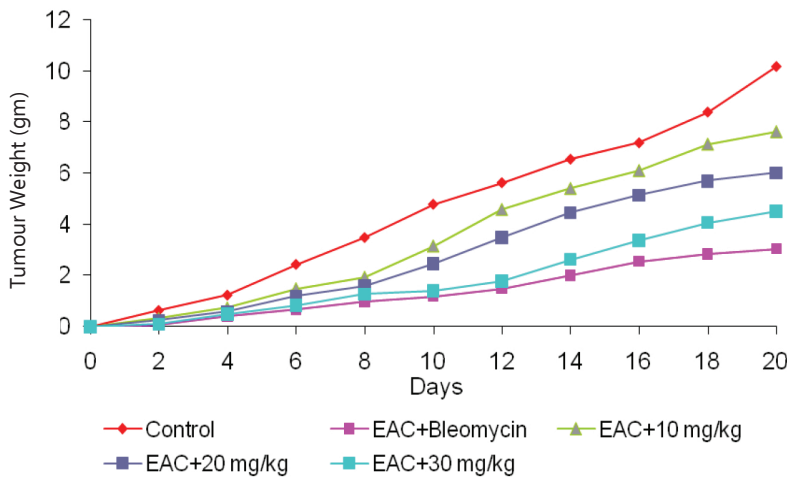
Treatment	Dose (mg/kg) (i.p.)	Mean survival time mean \pm SEM (days)	% Increase in life span
Control (untreated EAC cell-bearing mice)	-	23 \pm 0.98	-
EAC + Bleomycin	0.3	43 \pm 0.86***	86.95
EAC + BTSC	4	31 \pm 0.88*	34.78
	8	34 \pm 1.09**	47.82
	16	41 \pm 0.99**	78.26
EAC + PAS	10	27 \pm 1.45*	23.53
	20	30 \pm 2.25**	38.82
	30	33 \pm 1.5***	55.29

Data are expressed as the mean of results in six mice \pm SEM. Treatment was continued for 10 consecutive days. Significance values were * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ when compared with the control.



Data were expressed as the mean of results in six mice. Treatment was continued for 10 consecutive days.

FIGURE 1: Effect of BTSC on average tumor weight.



Data were expressed as the mean of results in six mice. Treatment was continued for 10 consecutive days.

FIGURE 2: Effect of PAS on average tumor weight.

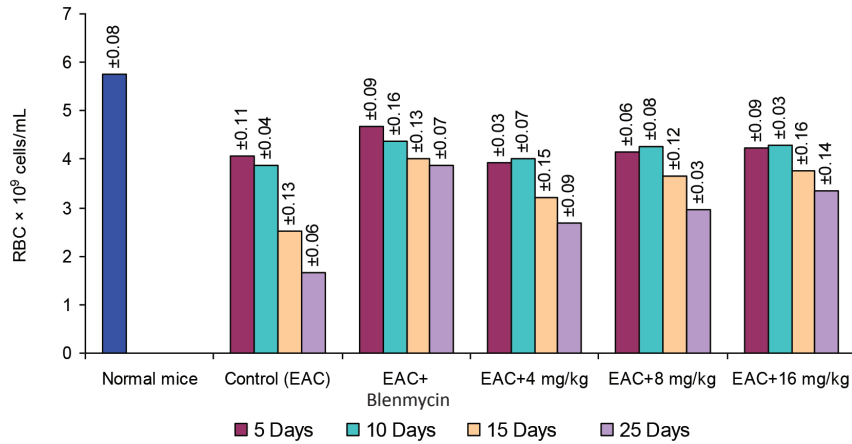
The treatment with Schiff bases also reduced the rate of tumor growth. At day 20, BTSC at the dose of 16 mg/kg (i.p.) and PAS at the dose of 30 mg/kg (i.p.) reduced the tumor weight by 65.05% and 55.70%, respectively, compared with the control mice. Bleomycin at the dose of 0.3 mg/kg (i.p.) caused a reduction by 73.83% (Figures 1 and 2).

The hematological parameters of both EAC cell-bearing mice and normal mice were examined. In EAC cell-bearing mice, all parameters (WBC, RBC, and hemoglobin content) were found to be significantly changed compared with those of the normal mice. The rate of deterioration, however, slowed down after the treatment (Figures 3–8). In case of parallel treatment of normal

mice, these parameters slightly changed from the normal values. After 25 days of the initial treatment, they were restored to normal values.

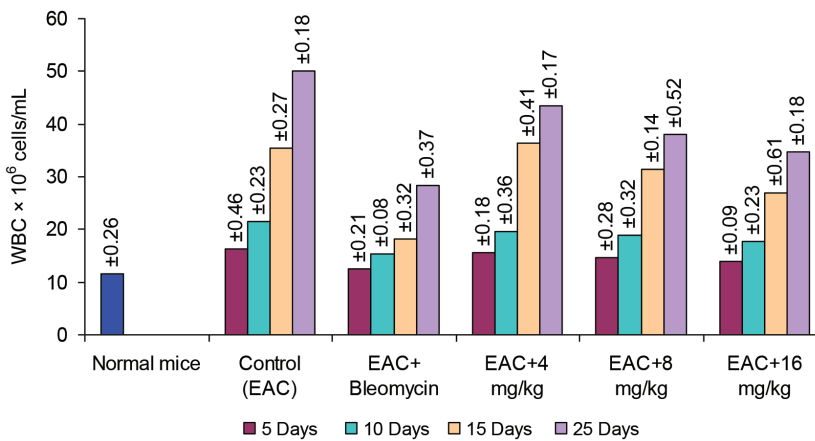
The effect of Schiff bases on the loss of transplantability of EAC cells was observed by the reduction of intraperitoneal tumor growth in mice re-inoculated with test compound-treated EAC cells (Table 4) compared with control. Maximum reduction (67.88%) of tumor growth was observed with BTSC at the dose of 16 mg/kg (i.p.), whereas PAS at 30 mg/kg (i.p.) reduced the transplantability of EAC cells by 58.03%.

The compounds at highest experimental doses also enhanced the number of both peritoneal cells and macrophages, to some extent, in normal mice (Table 5).



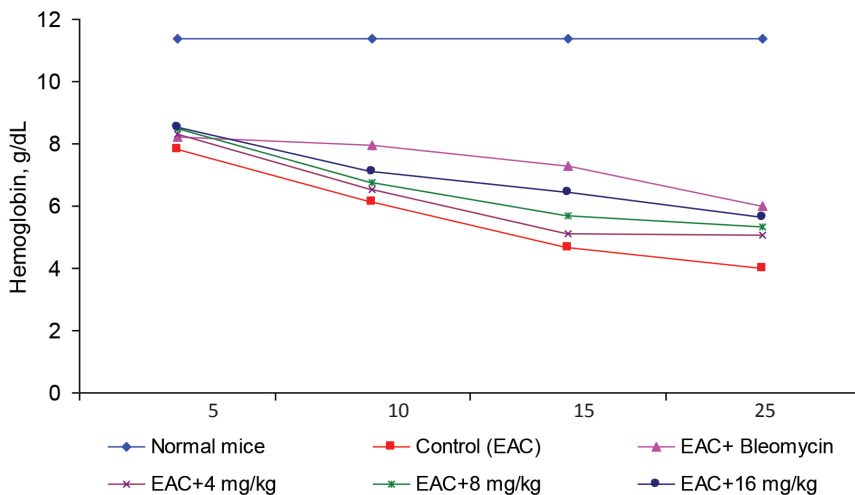
Data were expressed as the mean of results in four mice ± SEM. Treatment was continued for 10 consecutive days.

FIGURE 3: Effect of BTSC on RBC content of EAC-bearing mice on days 5, 10, 15, and 25.



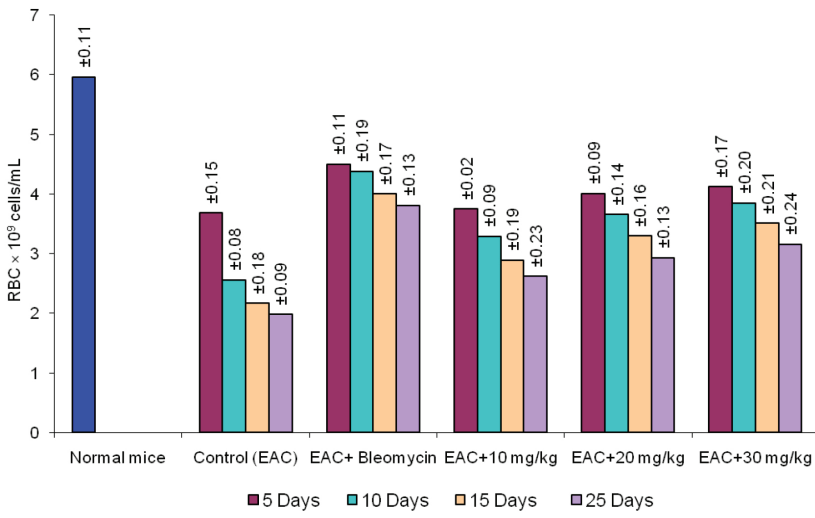
Data were expressed as the mean of results in four mice ± SEM. Treatment was continued for 10 consecutive days.

FIGURE 4: Effect of BTSC (4 mg/kg [i.p.], 8 mg/kg [i.p.], and 16 mg/kg [i.p.]) on WBC content of EAC-bearing mice on days 5, 10, 15, and 25.



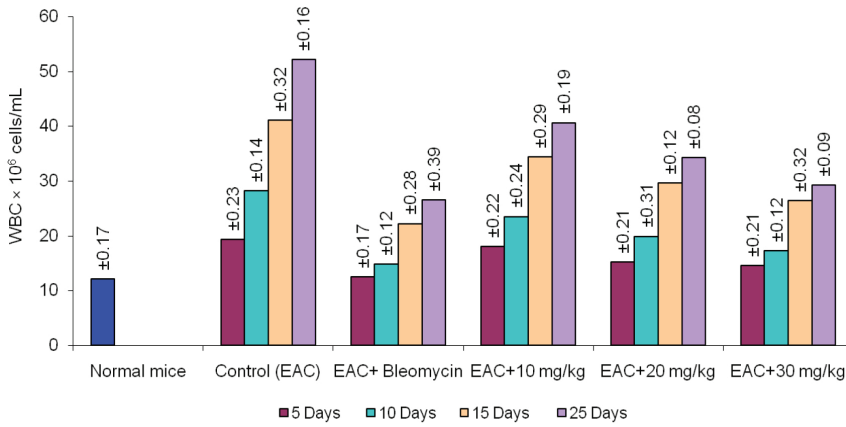
Data were expressed as the mean of results in four mice. Treatment was continued for 10 consecutive days.

FIGURE 5: Effect of BTSC on hemoglobin content of EAC-bearing mice on days 5, 10, 15, and 25.



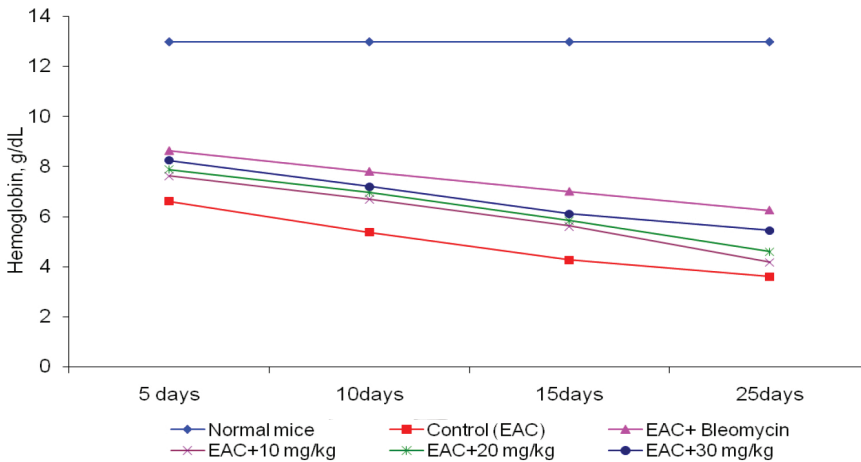
Data were expressed as the mean of results in six mice. Treatment was continued for 10 consecutive days.

FIGURE 6: Effect of PAS on RBC content of EAC-bearing mice on days 5, 10, 15, and 25.



Data were expressed as the mean of results in six mice. Treatment was continued for 10 consecutive days.

FIGURE 7: Effect of PAS on WBC content of EAC-bearing mice on days 5, 10, 15, and 25.



Data were expressed as the mean of results in six mice. Treatment was continued for 10 consecutive days.

FIGURE 8: Effect of PAS on WBC content of EAC-bearing mice on days 5, 10, 15, and 25.

TABLE 4: Bioassay of the Schiff bases.

Treatment	Dose (mg/kg i.p.)	No. of EAC cells ($\times 10^7$)	Cell growth inhibition on inoculating EAC cells with the drugs
Control (untreated EAC cell-bearing mice)	-	3.14 \pm 0.04	-
EAC + BTSC	16	1.01 \pm 0.007***	67.88%
EAC + PAS	30	1.32 \pm 0.05**	58.03%

Data were expressed as the mean of results in four mice \pm SEM. Significance values were **P < 0.01 and ***P < 0.001 when compared with the control.

TABLE 5: Effect of the test compounds on the enhancement of normal peritoneal cells in mice

Treatment	Dose (mg/kg i.p.)	Macrophages (cells/mL) $\times 10^6$	Total peritoneal cells $\times 10^6$
Control (normal)	-	1.44 \pm 0.42	3.82 \pm 0.29
Normal + BTSC	16	1.75 \pm 0.34**	4.26 \pm 0.16
Normal + PAS	30	1.86 \pm 0.21***	4.43 \pm 0.32

Data were expressed as the mean of results in four mice \pm SEM. Treatment was continued for three consecutive days. ***P < 0.001 and **P < 0.01 when compared with the control.

DISCUSSION

The results of the present study showed that the two Schiff bases were capable of reducing average tumor weight and increasing the life span of tumor-bearing mice. In all cases, these abilities increased with increased doses of the compounds. BTSC 16 mg/kg (i.p.) and PAS 30 mg/kg (i.p.) showed maximum values, which were quite comparable to those of bleomycin 0.3 mg/kg (i.p.).

Analogous results were obtained for EAC cell growth inhibition by these Schiff bases. With the increase in doses, the percentage of cell growth inhibition was found to increase noticeably.

All these results are considered to be very important and promising in justifying the potency of these compounds in cancer chemotherapy (21). The major problems usually encountered in cancer chemotherapy are myelosuppression and anemia (22, 23) due to the reduction of RBC and hemoglobin contents. This is probably owing to the deficiency of iron in hemolytic or myelopathic conditions (24). After treatment with each of the two synthesized compounds under investigation, all the hematological parameters were restored to normal. This can be inferred from the decreased rate of deterioration of

these parameters on treatment with these drugs (Schiff bases and bleomycin). The toxic effects of these Schiff bases are not very high. This indicates that both BTSC and PAS have protective actions on the hematopoietic system. The rectifying ability for the hematological parameters in EAC-bearing mice has been demonstrated by the increase in life span and other parameters studied for evaluating the potency of these compounds as antineoplastic agents. The effects of BTSC and PAS on the viability of EAC cells are found to be reduced significantly. BTSC shows a better effect. In addition, the treatment in normal mice increases the number of macrophages and peritoneal cells, which plays an important role in destruction of cancer cells by phagocytosis (25). Macrophages may produce some cytokines such as tumor necrosis factor (TNF) and interleukins inside the peritoneal cavity, which in turn may be responsible for killing tumor cells (26). Besides, high LD₅₀ values represent the low toxicity of the compounds to the host.

So, BTSC and PAS are expected to be effective anticancer agents with low toxicities. However, the information obtained from the present study is insufficient for establishing BTSC and PAS as novel anticancer drugs in clinical practice. Many more investigations

need to be carried out with these compounds using various other cancer cell lines and animals in order to substantiate the present findings.

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