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Antimalignancy Activity of *Bacillus thuringiensis* Serovar *Dakota* (H15) *in vivo*

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Abstract: *Bacillus thuringiensis* (*B.t.*) isolate, belonging to the serovar *dakota* (H15) produced non-insecticidal proteins crystallizing into bipyramidal-shaped parasporal inclusions. The activated parasporal inclusion proteins (CEBT-PP) showed *in vitro* preferential cytotoxicity to human and animal cancer cell lines. *In vivo* therapeutic assay was performed by challenging the *Bacillus thuringiensis* serovar *dakota* (H15) activated proteins at various concentrations ranged from 32.6-57.05 µg/mouse subdivided into equal doses 4-7 dose given at 4-7 successive days. The mean survival time (MST), medium day of death (MdDD) and duplicates of survival time (DST) were calculated. So, all of the above measurements had a direct relationship to the administrated doses of the activated proteins. The survival time was significantly increased ($P<0.05$) when compared with control positive untreated group. Also, the metastatic property of EAC was diminished and/or completely disappeared in the treated groups. The average tumor sizes and weights were estimated before and after death and revealed that the treated mice bearing EAC tumor can tolerate a large tumor mass which had grown during their longest survival time. These observations raise the presence of a new group of *B. thuringiensis* that may have anticancer activity and the possibility of new applications for the *B. thuringiensis* serovar *dakota* (H15) in the medical field.

*CEBT-PP: *Bacillus thuringiensis* serovar *dakota* (H15) parasporal inclusion activated proteins.

Key words: *Bacillus thuringiensis* serovar *dakota* · Parasporal inclusion proteins · Mammalian cells · Ehrlich Ascites Carcinoma · *In vivo* anticancer activity

INTRODUCTION

Bacillus thuringiensis (*B.t.*) was first isolated in Japan as pathogen of the sotto disease of the silkworm, *Bombyx mori* by *Ishiwata* early in the past century [1]. *B. thuringiensis*, is a gram-positive endospore-forming soil bacterium, produces large crystalline parasporal inclusions during sporulation. The inclusions often contain δ-endotoxin proteins that are highly and specifically toxic to agriculturally and medically important insect pests of several orders, including Lepidoptera, Dipetra and Coleoptera [2]. The mechanism of whereby insecticidal proteins from *B. thuringiensis* that exert their toxicity have been extensively studied and the generally accepted mechanism are as follows: In the insect midgut, the insecticidal parasporal proteins are solubilized under alkaline conditions and activated by proteases. Then the proteolytic fragments of the proteins bind to specific receptors on the surface of the apical brush border membrane of epithelial columnar cells, forming pores that disrupt cellular functions and finally kill the insects [3-10]. Many strains of *B. thuringiensis* isolated from a variety of natural environments some are producing insecticidal toxins while others do not. Thus a question appears for discussion is theses inclusion proteins have any antitumor activity?. Our previous study, provided a partial answer, it was found that a strains of *B. thuringiensis* var *dakota* that produced non-insecticidal inclusion proteins had preferential cytotoxicity against Human Acute Lymphocytic leukaemia T-Lymphocytes (ALL), Human Larynx Carcinoma cells (HEP-2), Human Lung Carcinoma cells (A549) and Ehrlich Ascites Carcinoma (EAC) *in vitro* where the inclusion proteins were cleaved to fragments by an appropriate protease forming CEBT-PP [11, 12]. Although few researchers around the world found cancer killing activity in such non-insecticidal parasporal inclusion *in vitro* like [11-19] however, they did not report any therapeutic trials *in vivo*. *Prasad* and *Shethna,* [20,

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21] tested *Bacillus thuringiensis* parasporal inclusion proteins as anticancer agent *in vivo* and they reported that the proteinaceous crystals of *B. thuringiensis* var. *thuringiensis* inhibits the growth of Yoshida Ascites Sarcoma (YAS) and Yoshida Sarcoma (YS) in rats and they referred this antitumor activity either to the long lasting antitumor immunity or to its ability to alter the cellular permeability of the tumor cells to macromolecules. However Rao *et al.* [22] studied the *B. thuringiensis* δ-endotoxin inhibitory effect on 6:12-dimethylbenzo 1:2-b:4:5-b`-dithionaphthene induced fibrosarcoma in Swiss mice and suggested that *B. thuringiensis* δ-endotoxin probably bring about tumor growth inhibition as a result of cytotoxic action on the tumor cells. These results was confirmed by Zamola *et al.* [23] how reported that irradiated spore-endotoxin mixtures from *B. thuringiensis* cultures induced antitumor effects, expressed as survival rates of C57 BL/6 mice inoculated with *Lewis* mouse lung carcinoma and subjected to treatments 24hr, later depended on the number of doses of the preparations administered (mixture and separated components).

No previous record for the antitumor activity of *B. thuringiensis* serovar *dakota* parasporal inclusion proteins against EAC in mice was recorded. So, this study aims at studying the Preliminary testing of the therapeutic effect of *B. thuringiensis* serovar *dakota* (H15) proteolytically activated proteins (CEBT-PP) on EAC bearing mice as a model of virulent malignant cancer cells in mammals.

***CEBT-PP:** *Bacillus thuringiensis* serovar *dakota* (H15) parasporal inclusion proteolytically activated proteins.

MATERIALS AND METHODS

Bacterial Strain: The *Bacillus thuringiensis* isolate, serovar *dakota* (H15) that used in this study was obtained from National Microbial Bank (Faculty of Agriculture, Ain Shams University, Egypt). It was grown at 28°C on nutrient agar (pH 7.6) consisting of meat extract (10 g), polypeptone (10 g), NaCl (2 g), agar (15 g) and distilled water (1000 ml). The sporulated cultures were harvested and washed three times in 1M NaCl and resuspended in distilled water. Parasporal inclusions were purified by biphasic separation technique by using Na_2SO_4 and carbon tetrachloride according to Pendleton and Morrison [24]. The purity of the isolated parasporal inclusion was checked by phase-contrast microscope. Purified inclusions were washed three times with distilled water and stored at 20°C until use.

CEBT-PP Preparation

CEBT-PP: *Bacillus thuringiensis* serovar *dakota* (H15) parasporal inclusion activated proteins was prepared as mentioned by Gad El-Said *et al.*[11,12]. Solubilization of the purified inclusions was performed according to Lee *et al.*[16] in 50mM Na_2CO_3 (pH10) at 37°C for 1h in the presence of 10mM dithiotheritol (DTT) and 1mM ethylene diamine tetrachloroacitic acid (EDTA). The solubilized inclusion solution was adjusted to pH 8 by 1N HCl. For analysis of the proteolytic processing (proteolytic activation), solubilized proteins were treated with proteinase K (final concentration: 30ìg/1.2 mg inclusion proteins/ml) at 37°C for 90 min. After incubation, 1mM phenylmehylsulphonyl fluoride (PMSF) “Sigma” was added to stop the proteolytic processing and its therapeutic activity was examined after their dialysis against solubilizing buffer.

Protein Determination: Protein concentration was measured by the method of Bradford [25] using bovine serum albumin as standard.

Experimental Animals: Non-inbred Swiss albino mice 60 day old, weighing approximately 28 gm obtained from the breeding unite of National Cancer Institute (NIC)-Cairo University. The animals were housed in suitable cages, atmosphere and kept in standard pellet diet and water. The diet consisted of not less than 20% protein, 5% fibers, 3.5% fat and 65% ash and supplied with vitamins (ad. Libitium).

Cell Lines: Ehrlich Ascites Carcinoma cells (EAC).These cells were supplied kindly from National Cancer Institute (NCI), Egypt.

Short-Term Viability Assays: In this study Short-term viability-assay using trypan blue according to Ian Freshney [26] to measure the proportion of viable cells inoculated in the tested mice.

Antitumor Activity of *Bacillus thuringiensis* Serovar *Dakota* (H15) Activated Proteins (CEBT-PP) in Experimental Cancer in Mice Bearing EAC:

The preliminary therapeutic activity of CEBT-PP was carried out according to Zamola *et al.* [23]. In order to evaluate the therapeutic effect of CEBT-PP on the survival and the growth of EAC tumor induced in the mice through different parameters as survival time, tumor size and tumor metastasis. A total of 35 Swiss albino mice (60 day old) and weighing approximately 28gm/each were used in 7 groups 5 mice each. Received at day zero *s/c* inoculation of $0.5X10^6$ EAC cells/mouse in 0.3 ml phosphate buffer saline (PBS).

Group I: Normal control group received no treatment or EAC cells.

Group II: Control negative received no EAC cells but inoculated with a maximum treatment dose at day 1 of the experiment (7 dose each 8.15ìg/ml/mouse, *s/c* for seven successive days).

Group III: Control positive (EAC bearing mice) inoculated with 0.3ml *s/c* at the thigh region of 0.5 X 10^6 EAC cells/mouse at the day zero of the experiment.

Therapeutic Assay: Mice Inoculated with Tumor Cell Line and Treatment:

Group IV: Inoculated with 0.3ml *s/c* of 0.5×10^6 EAC cells/mouse at the thigh region at day 1 of the experiment, they received four doses of treatment each was 8.15ìg /ml for four successive days 1ml/day/mouse *in situ*.

Group V: Inoculated with 0.3ml *s/c* of 0.5 X 10^6 EAC cells/mouse at the thigh region at day 1 of the experiment, they received five doses of treatment each was 8.15ìg /ml for five successive days 1ml/day/mouse *in situ*.

Group VI: Inoculated with 0.3ml *s/c* of 0.5 X 10^6 EAC cells/mouse at the thigh region in day 1 of the experiment, they received six doses of the treatment each was 8.15ìg /ml for six successive days 1 ml/day/mouse *in situ*.

Group VII: Inoculated with 0.3ml *s/c* of 0.5 X 10^6 EAC cells/mouse at the thigh region in day 1 of the experiment, they received seven doses of the treatment each was 8.15ìg/ml for seven successive days 1 ml/day/mouse *in situ*.

During the experiment period the apparent average tumor size was calculated in order to monitor the development of tumor size in the treated groups and the control positive group and to estimate the variation between them. A postmortem examination was performed for each dead animal in order to determine the real size of developed tumor, proliferation profile and its ability to metastasis in other areas. At the end of this experiment the survival time was estimated for each group in order to evaluate the potency of CEBT-PP proteins as anti-tumor, *in vivo*.

Statistical Analysis: The statistical analysis was performed according to Bailey [27].

RESULTS

Anti-Malignancy Activity of Activated Proteins (CEBT-PP) on the Mice Bearing EAC Cells: Regarding to Table 1, it is obvious that the relative survival percentage was directly proportionally increased with the elevation of the received total activated proteins amount from 32.60 to 57.05 µg/mouse subdivided in an equal amounts. It is also, obvious that the highest dose given (57.05µg/mouse) did not induce any mortality or even pathological changes in control negative group (group II) which means that it is tolerable dose (safe dose). In the meantime it is noticeable that these doses cause 20% relative survival percent in cancer bearing mice (group VII). Also, Table 2 revealed that the mean survival times (MST) of the treated mice bearing Ehrlich Ascites Carcinoma cells (EAC), range from 17 to 52 days which increased according to the number of injected doses, at the same time the mean survival time of group III mice injected with EAC is only 15 days. Comparing the untreated group III (control positive) with the groups that treated with CEBT-PP proteins it is clear that the median days of death (MdDD) were arranged in an ascending manner 18 (7-28); 30 (9-73); 32 (14-78); 32 (20-100) in a direct relationship with the number of CEBT-PP injected doses. It is also obvious that the survival time of the treated groups was duplicated more than four times than the untreated groups.

A post-mortem examination was carried out on the dead mice during the experiment in order to compare the relative metastasis percentage of the tumor in treated groups (No. IV, V, VI and VII) and untreated control positive group (No.III) of mice in relation to the number of injected doses of CEBT-PP proteins. It is clear that the highest lung metastasis percentage was recorded in the untreated group (group III) (80%) while it was significantly decreased in group IV (40%) and group V (20%). In the meanwhile groups no. VI and VII are free from any macroscopic lung metastasis, (Table 3 and Fig. 2). At the same time, the highest intra-abdominal infiltration of Ehrlich Ascites Carcinoma (EAC) was recorded in group III (60%) while groups V, VI and VII are free from any intra-abdominal infiltration (Table 3 and Fig. 2). So it is recorded herein that there is a reverse relationship between the incidence of metastasis either in the lung or in the intra-abdominal infiltration and the number of injected doses of CEBT-PP proteins.

Table 1: Comparison of relative survival percentage of Swiss albino mice injected with 0.5×10^6 Ehrlich Ascites Carcinoma cells (EAC) *s/c,* treated with CEBT-PP proteins

			No. of days post-inoculation										
Group No	No. of doses	Total protein in µ/ mouse	10	20	30	40	50	60	70	80	90	100	110
			Relative survival percentage										
I	-	-	100	100	100	100	100	100	100	100	100	100	100
II	7	57.05	100	100	100	100	100	100	100	100	100	100	100
III	-	-	60	40	-	-	-	-	-	-	-	-	-
IV	4	32.60	80	40	-	-	-	-	-	-	-	-	-
V	5	40.75	80	60	60	20	20	20	20	-	-	-	-
VI	6	48.90	100	80	60	40	20	20	20	-	-	-	-
VII	7	57.05	100	100	60	40	40	40	40	40	20	20	20

GpI: Normal healthy control.

GpII: Control negatives injected with 8.15ìg of CEBT-PP proteins /mouse/day for 7 successive days s/c.

GpIII: Control positive injected only with (EAC) cells.

GpIV: Treated with 8.15ìg of CEBT-PP proteins /mouse/day for 4 successive days s/c.

GpV: Treated with 8.15ìg of CEBT-PP proteins /mouse/day for 5 successive days s/c.

GpVI: Treated with 8.15ìg of CEBT-PP proteins /mouse/day for 6 successive days s/c.

GpVII: Treated with 8.15ìg of CEBT-PP proteins /mouse/day for 7 successive days s/c.

Table 2: Comparison between the mean survival time (MST), median day of death (MdDD) and duplicates of survival time (DST) in Swiss albino mice carrying Ehrlich Ascites Carcinoma (EAC) cells and treated with CEBT-PP proteins

Group No.	No. of received doses	Total protein in µg/mouse	MST	MdDD (Range of death day)	DST
I	-	-	All animals alive on day 100		
II	7	57.05	All animals alive on day 100		
III	-	-	15.4	18 (5-26)	-
VI	4	32.60	17.8 (NS)	18 (7-28)	1.15
V	5	40.75	32.6(NS)	30 (9-73)	2.11
VI	6	48.90	38.2 (S)	32 (14-78)	2.48
VII	7	57.05	52 (S)	32 (20-100)	4.37

GpI: Normal healthy control.

GpII: Control negatives injected with 8.15ìg of CEBT-PP proteins /mouse/day for 7 successive days *s/c*.

GpIII: Control positive injected only with (EAC) cells.

GpIV: Treated with 8.15ìg of CEBT-PP proteins /mouse/day for 4 successive days *s/c*.

GpV: Treated with 8.15ìg of CEBT-PP proteins /mouse/day for 5 successive days *s/c*.

GpVI: Treated with 8.15ìg of CEBT-PP proteins /mouse/day for 6 successive days s/c.

GpVII: Treated with 8.15ìg of CEBT-PP proteins /mouse/day for 7 successive days s/c.

S: Significant change in comparing with control positive ($P<0.05$).

NS: Non-significant change in comparing with control positive ($P\geq0.05$).

Table 3: Effect of CEBT-PP proteins on Ehrlich Ascites Carcinoma (EAC) metastasis in treated and untreated groups of mice

			Relative metastasis percentage	
Group No.	No. of doses	Total protein in µg/mouse	Lung metastasis	Intra-abdominal infiltration
I	-	-	-	-
II	7	57.05	-	-
III	-	-	80%	60%
IV	4	32.60	40%	20%
V	5	40.75	20%	0%
VI	6	48.90	0%	0%
VII	7	57.05	0%	0%

GpI: Normal healthy control.

GpII: Control negatives injected with 8.15ìg of CEBT-PP proteins /mouse/day for 7 successive days *s/c*.

GpIII: Control positive injected only with (EAC) cells.

GpIV: Treated with 8.15ìg of CEBT-PP proteins /mouse/day for 4 successive days *s/c*.

GpV: Treated with 8.15ìg of CEBT-PP proteins /mouse/day for 5 successive days *s/c*.

GpVI: Treated with 8.15ìg of CEBT-PP proteins /mouse/day for 6 successive days s/c.

GpVII: Treated with 8.15ìg of CEBT-PP proteins /mouse/day for 7 successive days s/c.

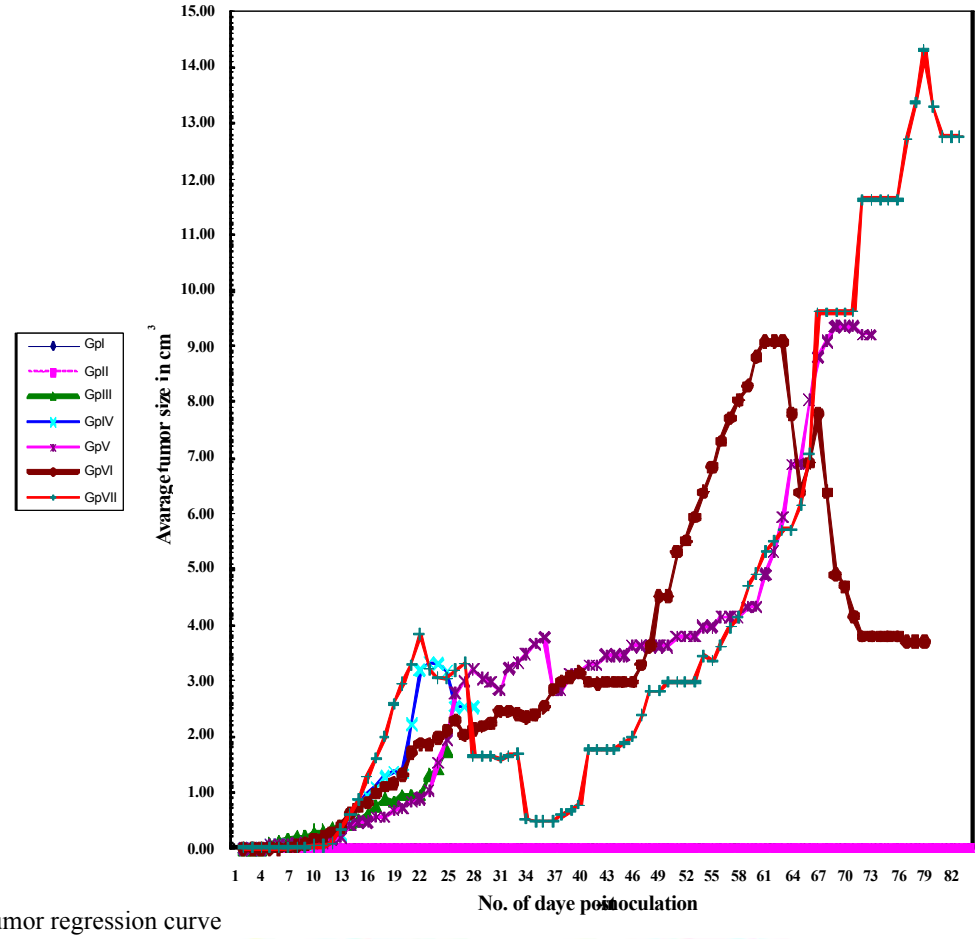

Fig. 1: Tumor regression curve

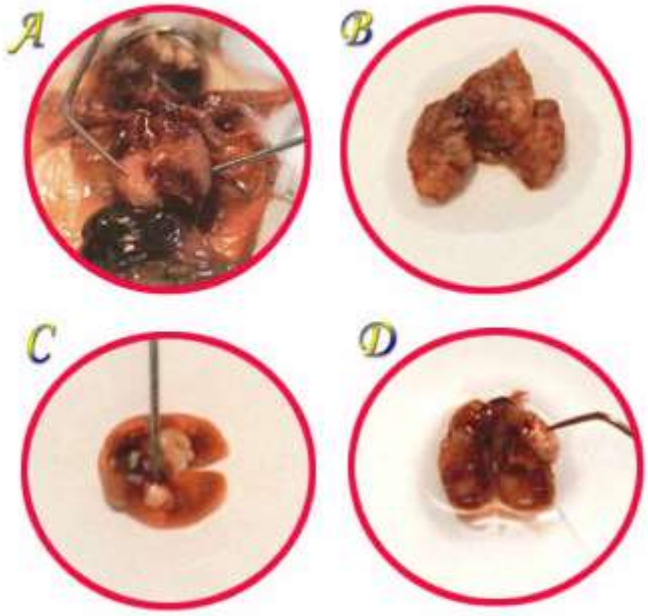

Fig. 2: Post-mortem examination of untreated Swiss albino mice bearing EAC shows lung metastasis (pointer end) in the form of creamy white small nodules distributed all over the lung lobes and gives marbling appearance (A and B). Sometimes merged together to form solid large tumor mass occupied the whole lung lobe(C and D).

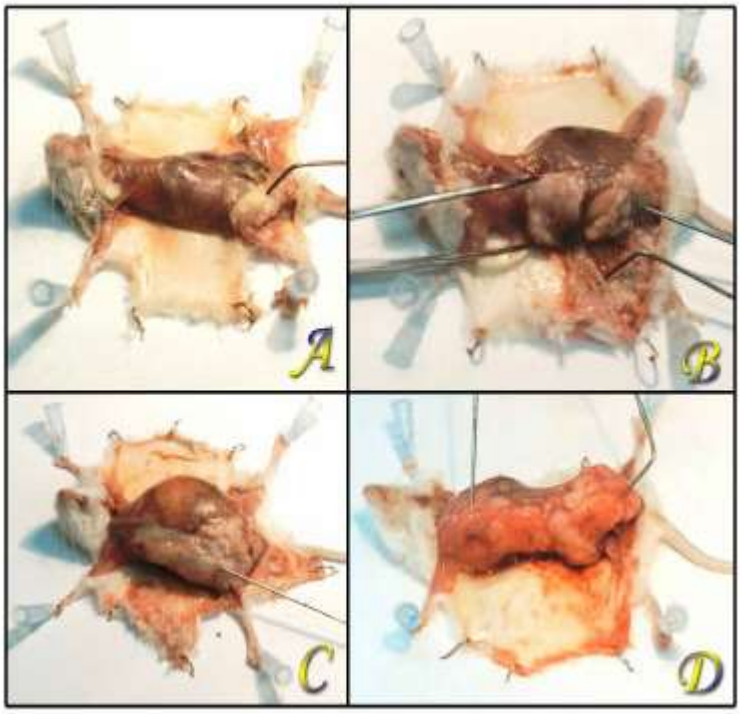

Fig. 3: Post-mortem examination of treated Swiss albino mice bearing EAC (A and B) shows moderate size of tumor masses. Treated mice groups show larger tumor mass (C and D).

Table 4: Post-mortem average tumor size (ATS) and weight (ATW) in Swiss albino mice bearing Ehrlich Ascites Carcinoma cells (EAC) in relation to the number of injected doses of CEBT-PP proteins

Group No.	No. of doses	Total protein in µg/mouse	Average tumor size (ATS) (± SE)	Average tumor weight (ATW) (± SE)
I	-	-	-	-
II	7	57.05	-	-
III	-	-	01.17 (± 0.38)	1.03 (±0.22)
IV	4	32.60	3.59 (±1.55) (S)	3.41 (±1.63) (S)
V	5	40.75	3.91 (±2.52) (S)	3.2 (±2.01) (S)
VI	6	48.90	1.93 (±0.29) (NS)	1.81 (±0.43) (NS)
VII	7	57.05	2.94 (±0.66) (S)	3.17 (±0.79) (S)

GpI: Normal healthy control.

GpII: Control negatives injected with 8.15ig of CEBT-PP proteins /mouse/day for 7 successive days *s/c*.

GpIII: Control positive injected only with (EAC) cells.

GpIV: Treated with 8.15ig of CEBT-PP proteins /mouse/day for 4 successive days *s/c*.

GpV: Treated with 8.15ig of CEBT-PP proteins /mouse/day for 5 successive days *s/c*.

GpVI: Treated with 8.15ig of CEBT-PP proteins /mouse/day for 6 successive days s/c.

GpVII: Treated with 8.15ig of CEBT-PP proteins /mouse/day for 7 successive days s/c.

S: Significant change in comparing with con0trol positive ($P<0.05$).

NS: Non-significant change in comparing with control positive ($P>0.05$).

It is noticeable that, post-mortem average tumor size (ATS) and weight (ATW) are increased in treated groups (IV, V, VI and VII) in comparing to the untreated control positive group (III) after cessation of the treatment at day 7 (Table 4).The effect of treatment on the tumor size in EAC bearing Swiss albino mice repeatedly injected with fixed doses of CEBT-PP proteins is recorded in Table 4 and Fig.1. Where the tumor growth exhibited two definite stages in treated mice and one stage in the untreated control positive mice (group III). The first stage (drug administration phase) was extended from the 1st to 7th day post-inoculation of Ehrlich Ascites Carcinoma (EAC) cells and characterized by inhibitory effect of the activated protein on the growth rate of the tumor. At the end of this stage (cessation of treatment) the tumor starts to grow normally in all treated animals. While the untreated control

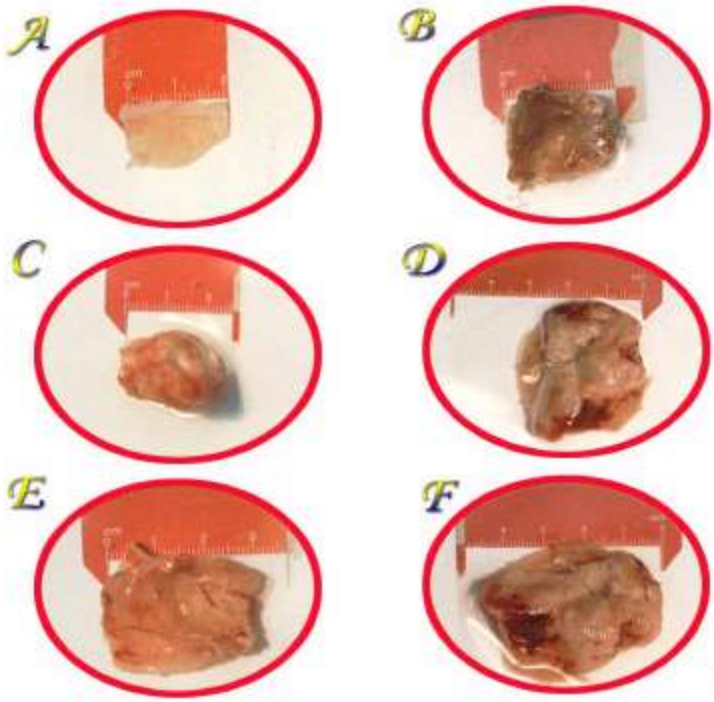

Fig. 4: Post-mortem examination of untreated control positive Swiss albino mice bearing EAC (A and B) shows moderate size tumor masses at the site of inoculation ranged from 2-2.5cm in diameter. The treated groups show relatively larger tumor masses at the site of inoculation ranged from 2.5-4cm in diameter (C and D and E and F).

positive group (group III) was recording accelerating growth rate of the tumor in this phase then died completely at day 26 due to either lung metastasis and/or intra-abdominal infiltration in the majority of animals as shown in Table 4 and Figs. 2 and 3.

The second stage (drug cessation phase) was extended from day 8 to day 82 post-inoculation of EAC cells and characterized by stagnation of the tumor growth rate in groups V, VI and VII and complete disappearance of the tumor in 20% of mice in group (VII) at day 40 (Table 4 and Fig. 1). This stage was ended with death of all animals' in-groups V, VI; however, a surprising result was recorded in group VII, when 20% of group VII survived until the end of the experiment (Table 4 and Fig. 1).

DISCUSSION

Previous study, Gad El-Said *et al.* [12] arises an issue here, if that selective cytotoxicity of the activated proteins (CEBT-PP) to the tumor cells *in vivo* was the same as that happened *in vitro* or not?. To answer this question a few remarks must be mentioned like, a measurement of cytotoxicity *in vitro* is a purely cellular event as presently carried out. It would be very difficult to re-create the complex pharmacokinetics of drug exposure, for example, *in vitro*; there will be significant differences in drug exposure time and concentration, rate of change of concentrations, metabolism, tissue penetration, clearance and excretion [26]. Many nontoxic substance become toxic after metabolized by the liver; in addition, many substances that are toxic *in vitro* may be detoxified by liver enzymes [26]. For testing *in vitro* to be accepted as an alternative to animal testing, it must be demonstrated that potential toxin reach the cells *in vitro* in the same form as they would *in vivo* and this may require additional processing by purified liver microsomal enzyme preparations [28] or Co-culture with active hepatocytes [29]. The nature of the response must also be considered carefully. A toxic response *in vitro* may be measured by changes in cell survival or metabolism, while the major problem *in vivo* may be a tissue response, e.g., an inflammatory reaction or fibrosis and this problem can not be dissolved unless a model of these response would be constructed perhaps by utilizing cultures resembled from several different cell types and maintained in the appropriate hormonal milies and this is impossible to achieve until now.

For the sake of all above mentioned problems that hindering or interfere the truthful evaluation of these cytotoxic proteins as anticancer agent, it was necessarily

to test these cytotoxic proteins against mice bearing cancer model, in order to evaluate this activated proteins as an anticancer agent and to know their mode of action. So, they must face the *in vivo* challenge including its standard known parameters. In this study many vital and important results were achieved including survival time and reduction of the metastatic ability of implanted cancer cells (EAC). An extraordinary notes were observed, that the relative survival percentage, mean survival time, median day of death and duplicates of survival time were in direct relationship with the doses of the CEBT-PP proteins administer in the tumor bearing mice (Table 1 and 2) where the control positive group died at day 27 while group VII which received 57.05µg activated proteins/mouse, divided equally on seven successive days, continued until the end of the trial recording 20% survival percent, also the mean survival time in the treated groups was higher than that recorded in the control positive group as it even reached 4.3 time of that recorded by control positive group. These results agree with those reported by Prasad and Shethna [20, 21] and Rao *et al.* [22]. However, the treated non-bearing tumor mice (control negative) tolerate the same dose (57.05µg activated proteins /mouse divided equally on seven successive days) with no clinical manifestation and maintaining their normal vital functions till the end of the experiment and even after that.

In addition to all above mentioned notices, it was found that the metastatic properties of EAC were diminished or completely reduced only in the treated groups of mice bearing EAC, either lung metastasis or intra-abdominal infiltration. While they were the main feature accompanied the control positive group as lung metastasis reached up to 80% of control positive group while intra-abdominal infiltration recorded 60% of control positive group (Table, 3) (Fig. 2 and 3). These results suggest that the CEBT-PP proteins succeeded in one of most important therapeutic parameters of anticancer agent which is the reduction of the metastatic properties of the malignant tumor, (EAC) and this was evidently in direct relationship with the administered dose of activated proteins. But a query still exists, why the metastatic properties of EAC were diminished within the treated groups?. This question is really arduous to reply but little observations may be of benefit to partially clarify this question.

- These results revealed that the activated proteins were effective in the prevention of tumor metastasis and this acts in dose dependent manner.
- The significant role played by the proteolytic enzymes in the invasive behavior of the embryonic cells has been known for many years [30].
- A direct correlation between invasive and metastatic ability and the production of MMPs metalloproteases by cancer cells was documented several years ago [31-36].
- In our previous study [11 and 12] it was cleared that the activated parasporal inclusion proteins (CEBT-PP) especial 29 kDa act as metalloproteinases inhibitor that prevent metastasis of the injected tumor (EAC) in the treated groups, particularly group (VI and VII). These results presented herein indicate that in addition to *in vitro* cancer cells killing activity of the CEBT-PP proteins, these proteins (particularly 29 kDa) act as metalloproteinases inhibitor in the treated mice. Where metastasis of the tumor was effectively prevented in group (VI and VII) and complete regression of the tumor was recorded in 20% of group VII.
- The complete regression and/or localization of tumor in advanced stages by CEBT-PP proteins are of value because this type of tumor is highly metastasized.

These results are in agreement with those obtained by Kwa *et al.* [37], Mizuki *et al.* [38] and Akao *et al.* [39]. So, this CEBT-PP protein may be of value as presurgical interference directed to diminish the metastatic activity of tumor cells. It is also noteworthy, that the tumor size in lived mice bearing EAC showed significant regression of the tumor during the course of the treatment whenever the drug was administered compared to the control positive group and start to grow thereafter cessation of the treatment (Table 4).Although by careful analysis of the growth cycle it was cleared that the growth cycle passed through two definite phases, the first phase (drug administration phase) extended from day one to day seven, seemed to be of a common feature of slow continuous growth of the tumor in control positive group while a significant reduction in tumor size in treated groups was recorded. This finding is most prominent in group VII that received the highest dose of the activated proteins (Fig 1).

The second phase (drug cessation phase) started from day 8 to day 82 approximately, the tumor size started again to grow spontaneously in treated and untreated group. Surprisingly after cessation of drug administration, treated mice bearing EAC can tolerate a large tumor mass that continuously grown during their longest survival time with diminished metastatic properties (Fig. 4).

The conflicting and unexpected result regarding to the tumor size in treated mice bearing EAC cells after cessation of treatment strongly suggest that the parasporal activated proteins elicits its inhibitory effect on tumor bearing mice only when it is in proximity with tumor cells. This was further substantiated by the observation that the growth rates and tumor size that started again after cessation of treatment. Thus, the ability of the toxin to prevent growth of the tumor appeared to be probably due to direct killing of the tumor cells, Rao *et al.* [22]. These results give an idea about the mode of action of CEBT-PP proteins *in vivo* as it could be attributed to the direct cytotoxicity due to the presence of specific receptors on cancer cells membranes. So, from the above mentioned notices it is clear that CEBT-PP had a potent tumor inhibitory activity during the treatment course and act in a drug administration dependent manner. The effectiveness of the split dose administration may be required due to the rapid removal of the protein from the system. The detoxification of the protein after inoculation may also be the reason for the progressive enlargement of the tumor size. These results suggest that continuous exposure of the tumor to the activated proteins is necessary for complete curing and this is usual with all anticancer drugs as we deal with an active uncontrollable idiopathic fatal disease and this suggestion is in agreement with those obtained by Prasad and Shethna [21].

Overall, such work may still offer another modality of cancer therapy, though definitive assessment must wait until these observations are clarified through introduction of these activated proteins to medical and pharmaceutical laboratories as a novel tumiricidal agent derived from prokaryotic cells for further studies.

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