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Bioflocculants Produced by Isolated Bacteria from Egyptian Soil II-Cytopathological Effect of Extracellular and Intracellular Bacterial Extracts

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Abstract: Genus *Bacillus* is useful not only for the production of commercially bio-molecules (insecticides, enzymes, antibiotics, antitumor, pesticides, etc.), but also as a source of spoilage or pathogenic organisms transmitted through foods and beverages. *Bacillus cereus, Bacillus thuringiensis* were isolated and identified in our previous study. The possible histopathological effects caused by extracellular bacterial bioflocculants (glycoproteins) against biological systems in rats (blood, liver, kidney and brain) were studied. The cytotoxic effect of intracellular proteins (parasporal proteins) on tumor and normal cell lines and their abilities to selectively target cancer cells without affecting normal cells were also investigated. The experimental animals (60 young rats) were treated orally daily for 90 days: group I was regarded as control received only tap water, group II and III were given tap water containing extracellular bacterial bioflocculants of *Bacillus cereus* (B1) and *Bacillus thuringiensis* (B2) at a dose of 35 ppm (3.5mg/kg b.wt), group IV was given tap water containing Aluminum salts at the same previous dose. The results showed that loss of weight in Group IV, similar weight of Group II and weight gain in Group III when compared to Group I (control). The results also revealed that slightly histological changes which observed in liver, kidney and brain in the rats which received Aluminum salts in the group IV as compared to control, while no significant changes was observed in groups II and III which received the tested bioflocculant and these results were insured by the tested biochemical tests. Moreover, two cancer cell lines were used in this study to investigate the antitumor effects of intracellular proteins of *Bacillus cereus* and *Bacillus thuringiensis*. Furthermore, we used fibroblast cells (MRC5) of human lung as a control. The parasporal proteins showed preferential cytotoxicity to human and animal cancer cell line *in vitro*. Parasporal proteins revealed dose dependent cytotoxicity against two tumor cell lines with lethal concentration of 4.36 and 10.87µg/ml, respectively. The cytopathic effect induced by *Bacillus cereus* and *Bacillus thuringiensis* parasporal protein were characterized by remarkable kariolysis of the nucleus, cell ballooning, loss of cell membrane integrity ended with complete cell lyses. The two strains belonged to the same family and have two types of proteins that have no cytotoxic effect on normal cells.

Key words: Bioflocculants · Rats · Biochemical tests · Histological changes · Liver · Kidney and Brain · Parasporal protein · Cell lines · Anticancer agents

INTRODUCTION

In general, it is known that the genus of *Bacillus* includes a variety of medical and industrially important species and has a history of safe use in food, medicine and industry. The members of genus *Bacillus* encompass a great diversity of bacterial species and have a ubiquitous distribution in the environment. Because of

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their resistant spores and the capacity of vegetative cells to secrete a wide variety of enzymes, this genus is important not only for the production of commercially important bio-molecules (insecticides, enzymes, antibiotics, antitumor, pesticides, etc.), but also as a source of spoilage or pathogenic organisms transmitted through food and beverages [1]. *Bacillus cereus, Bacillus thuringiensis* are members of the *Bacillus cereus* group of bacteria. They differ widely in their phenotypes and pathological effects. *Bacillus cereus* which is ubiquitous in the environment and acting as a source of spoilage through air to food samples needs suitable discriminatory typing methods to facilitate epidemiological investigation. *B. cereus* causes food-borne disease syndromes associated with enterotoxin and emetic toxin. *B. thuringiensis* is an insect pathogen and it is widely used for the biological control of insects in crop protection [2]. It is interesting to find that the two species have included two types of proteins; extracellular bioflocculant (glycoproteins) that was used as a coagulant in raw water treatment instead of Aluminum sulfate and intracellular parasporal protein that were used as antitumor agent [3]. Aluminum salts are by far the most widely used coagulants in water treatment. However, several serious disadvantages of using Aluminum salts including Alzheimer's disease and similar health related problems associated with residual aluminum in treated waters have been identified. There is also a problem of reaction of Aluminum with natural alkalinity present in the water leading to a reduction of pH and a low efficiency in the coagulation process [4]. Aluminum is the third most abundant element and most common metal in the earth's crust. Exposure to Aluminum is almost inevitable, since it is present everywhere as an active substance or an additive. This element enters the human body via food, air, water and drugs and is present in many manufactured foods such as processed cheese, baking powders, cake mixes, frozen dough, pancake mixes and pharmaceutical products [5]. In recent years, various epidemiological studies have suggested that Aluminum plays a pathogenic role in Alzheimer's disease [6, 7]; also Aluminum accumulates in kidney, brain and liver [5]. Besides the brain, Aluminum in high doses was also shown to damage the kidneys, bones, heart and lungs and unfortunately very little data exists for other organs [5]. One contemporary approach that had received attention was the probing of the bioactive substance isolated from natural sources. One of these approaches is the using of bacterial metabolites [8]. Bioflocculants mostly come from the natural secretions of microorganisms and cell lyses [9]. Furthermore, they are kinds of extracellular biopolymers of macromolecular substances such as proteins, glycoproteins, polysaccharides and nucleic acids [10]. The second type of protein of these bacteria is parasporal proteins that were used as anticancer agent. New approaches to cancer therapy that are tumor cell directed and specifically lethal to malignant cells and less toxic to normal tissues are being observed and developed. The anticancer agent used in present research is intracellular protein in nature and this is new as most of previously interested authors in cancer biotherapy focused on bacterial toxins of indole derivative [11] or Lipopolysaccharides (like those extracted from *Streptococcus pyogenes*) [12]. Mizuki *et al*. [13] were the first to attempt a large-scale screening of *B. thuringiensis* strains whose parasporal inclusion proteins are non-hemolytic but cytocidal to human cancer cells [14].

The aim of this study was to investigate the effects of extracellular proteins in biological system, which will give some idea about target organ of the toxicity *in vivo*, also to test intracellular proteins in cancer treatment *in vitro* against the frequently known cancer cell lines and their ability to discriminate between normal and cancer cells.

MATERIALS AND METHODS

In vivo Experiments

Experimental Animals: Healthy albino rats, three months age, weighting (169 to 196 g) was used as experimental animals in the present investigation. They were obtained from Animal House in National Center for Radiation and Research Technology, Cairo, Egypt. They were kept under observation during the period of experiment to exclude any intercurrent infection. The chosen animals housed in plastic cages under good aerated covers at normal atmospheric temperature (25±2°C) and photoperiod (14h light: 10h dark) cycles as well as under good ventilation. The animals received standard balanced diet and water. All animals' procedures are in accordance with the recommendations for the paper care and use of laboratory animals stated by the National Standards of Animal Care [15].

Chemicals and Doses:

Group I: Control group received only tap water.
Group II: received (glycoproteins) from *Bacillus cereus* in a dose 3.5 mg/l.
Group III: received(glycoproteins) from *Bacillus thurengeinsis* in a dose 3.5 mg/l.
Group IV: received Aluminum sulfate salts 3.5 mg/l of tap water.

Experimental Design: The sixty young animals were divided into four groups comprising fifteen animals in each and in such a way that mean body weights are equal and total weight variation should not exceed ± 20% of the mean, each designed as the following: All animals were given orally and daily the tested materials for 90 days. The tested materials were given as suspension mixed with tap water and control receiving tap water only. Before each experiment, the tested materials were further diluted to the desired final concentration. All preparations of materials were freshly prepared on the day of use. For all *in vivo* studies, all animals were observed for morbidity (rapid weight loss, dehydration, slow movement) and mortality twice daily. General clinical observations were made twice a day at the same time throughout study. The animals were observed for changes in weight, skin, fur, eyes and mucus membrane every two weeks from the beginning of experiment.

Determination of Hematological Biochemical Parameters: Blood samples were collected from each rat by venipuncture of the heart into EDTA-containing tubes and blain tubes, allowed to coagulate at room temperature then centrifuged at 3000 rpm for 20 minutes to obtain serum. The cell counts of the total hemoglobin, RBC's and RBC's indesis, WBC's and differential counts were determined by Cell-Dyn Sapphire hematology analyzer (USA). Biochemical analysis of the blood including Alanine aminotrasferase (ALT) and aspartate aminotransferase (AST) activities in the serum were determined according to Reitman and Frankel [16] creatinine, urea in the serum was determined according to Rojkin *et al.* [17].

Histological Investigation: On completion of experiments, animals were sacrificed under anesthesia on 91st day of the study and were subjected to a detailed gross histological examination: the liver, kidneys and brain were quickly removed, fixed in 10% buffered neutral formalin for 24 hours. The fixed organs were washed in tap water then dehydrated in ascending series of alcohol. Specimens were cleared in xylene and embedded in paraffin at 56°C in hot air oven for 24 hours. Paraffin bees wax tissue blocks were prepared for sectioning at 4–5µm, by rotary microtome and collected on glass slides, deparaffinized and stained with Harris hematoxylin and Eosin then examined through the light microscope [18].

***In vitro* Experiments:** The aim of this study to test intracellular proteins in cancer treatment *in vitro* against the frequently known cancer cell lines and their ability to discriminate between normal and cancer cells.

Tissue Culture Cell Lines: Ehrlich Ascites Carcinoma cells (EAC) were supplied kindly from National Cancer Institute (NCI), Egypt, Fibroblast human lung normal cells (MRC-5) was obtained from VACSERA, Egypt. All cell lines were maintained and propagate according to the instructions supplied from cell culture manufacturer.

Cytotoxicity Assay: A cell suspension of 100µl, containing $2x10^4/ml^{-2}$ viable cells was delivered to each well of microtiter plate and pre-incubated at 37°C for 24h in CO_2 incubator. The viable cell count was calculated by using trypan blue [19]. Then each well received 50µl of the fresh Dulbicos Modified Eagles Media (DMEM) and 50µl of parasporal proteins fraction solution in concentration ranging from 0.047 to 66.6 μgml^{-1}. Three replicates were used for each dilution. After incubation of the tested proteins with the tested cell lines at 37°C in CO_2 incubator, the cells were examined for cytotoxic effects at adequate intervals for 24h under inverted microscope. Two assays were carried out:

Trypane Blue Assays: Trypane blue was carried out according to Ian Freshney[19] to measure the proportion of viable cells.

Crystal Violate Assay: Crystal violate cytotoxicity assay was used because it is a most sensitive and advanced technique used in this field for evaluation of anticancer drug *in vitro* according to Mizuki *et al*. [20] as the following steps:

- Selected grown cell line was removed from the growth flask by trypsinization using 1ml of 0.25% Trypsin-EDTA for 2-4 min.
- Number of viable cells per ml was calculated by trypan blue (short-term viability assay).
- 2 X 10^4 cells per 100µl RPMI media without phenol red was added to each well of microtiter plate and pre-incubated at 37°C for 24h in CO_2 incubator.
- Ten µl of the tested sample was added to each well post-incubation.

- The tested plate was observed for 24h through regular intervals during its incubation at 37°C in CO_2 incubator and observed cytopathic effect (CPE) was recorded spontaneously.
- After 24h of incubation 10µl of crystal violate stock solution was added to each well and incubated for 3 to 4h at 37°C.
- At the end of incubation period the medium was removed and the converted dye was solubilized with 150µl of glacial acetic acid/well during shaking for 15-30 min.
- Absorbency of converted dye was measured (as OD) at a wavelength of 564nm, by means of ELISA reader.

Statistical Analysis: The obtained data were subjected to analysis of variance (ANOVA) test, followed by student's t-test to compare various groups with each other. Results were expressed as mean + standard error (SEM) and values of $p>0.05$ were considered statically non significant while $p<0.05$ and $p<0.01$ were considered significantly different and highly significantly different, respectively. All data in the text and tables are expressed as a percentage of dark control of at least three samples; experiments were repeated 3 times. The statistical analysis was carried out by GraphPad® Prism Software, (USA).

RESULTS

Effect of Extracellular Bacterial Extracts (glycoproteins) of the Tested Bacteria on Serum Enzyme Activities (*In vivo*)

Biochemical Parameters: Sixteen animals were included in this study. The clinical differences between groups are shown in Tables 1, 2 and 3. The results of the current study show variance in complete blood count (CBC) for all groups and also revealed no significant differences in liver enzymes ALT and AST and kidney function between control and group II and III that received the tested bioflocculant material (glycoproteins) from *Bacillus cereus* and *Bacillus thuringiensis,* respectively as shown in Figures 2a, 2b, 2c and 2d. These results were insured with histological examination as shown in Figures 3a, 3b, 3c, 4a, 4b 4c, 5a, 5b and 5c, also the results were insured with the weights of rats that were examined every two weeks from the beginning of experiment as shown in Table 1 and Fig. 1. On the other hand, Aluminum sulfate administered rats exhibited a highly significant excess in liver enzymes ALT and AST, also show clinical pathological effect in CBC and kidney function by time in compared with control as shown in Tables 2b and 3b and Figures 2a, 2b, 2c and 2d. These results were insured with histological examination as shown in Figures 6a, 6b and 6c.

Table 1: Mean of body weights of Rats during experimental periods

	Time						
Rat groups	Initial	2nd week	4th week	6th week	8th week	10th week	12th week
Control group	186	205	230	242	261	280	300
Bacillus thuringiensis	197	219	239	251	263	307	331
Bacillus cereus	164	179	201	220	257	277	284
Aluminum sulfate	170	185	199	214	229	243	259

From the above results it seems clear that the glycoprotein of *Bacillus thuringiensis* has increased rats weight during the term of the experiment.

Table 2a: Complete Blood Count (CBC)

Groups		Group (I)			Group (II)		
Parameters	Range	Mean ±SD	P value	Range	Mean ± SD	P value	Normal range
Hb g/dl	11-13.3	11.70±0.5	>0.05	10.9-13	11.6±0.8	>0.05	11-19.2
RBC,s X 10^{12}/L	7.75-8.1	7.895±0.2	>0.05	6.78-7.82	7.27±0.4	<0.0001	6.76-9.75
HCT%	42.3-49.8	45.32±3.3	>0.05	38.6-42.1	39.7±1.3	>0.05	39-59
MCV fL	55.5-56.9	56.15±0.6	>0.05	42.2-58	53.07±6.5	<0.0001	48-70
MCH pg	17-17.7	17.43±0.3	>0.05	17.2-17.8	17.53±0.3	>0.05	--
MCHC g/dl	38.9-39.9	39.43±0.5	>0.05	39.2-40.8	39.93±0.6	>0.05	40
PLT X10^9/L	380-407	396.3±9.3	>0.05	287-378	320±32	<0.0001	150-460
MPV fL	7.8-8.4	7.46±0.3	>0.05	7.6-7.9	7.73±0.2	>0.05	--
WBC,s X10^9/L	7-8.2	7.533±0.4	>0.05	6.8-7.7	7.217±0.3	>0.05	6-18
Lymph X10^9/L	51-59.4	53.4±3.1	>0.05	51.5-58.4	53.53±2.6	>0.05	65-85
Lymph %	34.2-40.9	37.85±2.6	>0.05	34.4-39.9	37.35±2.1	>0.05	4.78-9.12

Table 2b:

Groups		Group (III)			Group (IV)		
Parameters	Range	Mean ± SD	P value	Range	Mean ± SD	P value	Normal range
Hb g/dl	10.6-12.2	11.23±0.6	>0.05	10.3-11.1	10.63±0.4	<0.0001	11-19.2
RBC,s X 10^{12}/L	6.51-7.9	7.322±0.5	<0.0001	6.03-7.3	6.620±0.6	<0.0001	6.76-9.75
HCT%	39.9-49.6	43.9±4.4	<0.0001	26.5-33.5	30.9±2.9	>0.05	39-59
MCV fL	54.4-57.9	56.77±1.3	>0.05	43.5-45.9	45.05±9.8	<0.0001	48-70
MCH pg	17.2-17.8	17.47±0.3	>0.05	16.5-18.3	17.02±0.7	>0.05	--
MCHC g/dl	36.7-40.6	38.32±1.3	>0.05	38.5-43.3	41.65±1.7	>0.05	40
PLT X10^9/L	289-387	324.3±35	<0.0001	281-292	287.3±3.6	>0.05	150-460
MPV fL	7.2-7.6	7.46 ±0.5	>0.05	7.1-7.5	7.3±1.6	<0.0001	--
WBC,s X10^9/L	7.2-7.9	7.633±0.3	>0.05	4-5.5	4.783±0.6	>0.05	6-18
Lymph X10^9/L	48-56	52.22±3.1	>0.05	41.5-53.4	48.3±4.5	>0.05	65-85
Lymph %	32.1-41.3	37.15±4.1	>0.05	33.9-42.1	38.2±2.6	<0.0001	4.78-9.12

Table 2a and 2b: a clinical pathological effect in CBC in treated rats: Group (I): Control, Group (II): *Bacillus cereus*, Group (III): *Bacillus thuringiensis*, Group (IV): $Al_2 (SO_4)_3$. P value >0.05: no significant results in compared to control, P value <0.0001: significant results in compared to control. Hb: Hemoglobin, RBC's: Red blood cells, WBC's: White blood cells, HCT: Hematocrit, MCV: Mean corpuscular volume, MCHC: Mean corpuscular Hemoglobin conc., PLT: Platelet, Lymph.: Lymphocytes, MPV: Mean platelet value.

Table 3a: Blood chemistry

Groups		Group (I)			Group (II)		
Parameters	Range	Mean ±SE	P value	Range	Mean ±SE	P value	Normal range
ALT(SGOT)	90.3-102	97.52±4.1	>0.05	89.7-95.3	92.38±2.3	>0.05	88.1-147
AST(SGPT)	37.2-42.2	39.15±1.8	>0.05	39.1-45.3	42.22±2.5	>0.05	35-80
Urea	11-15	12.33±1.8	>0.05	13-19	15.17±2.4	>0.05	5-29
Creatinine	0.51-0.59	0.54±0.1	>0.05	0.48-0.62	0.56±0.1	>0.05	0.5-1

Table 3b:

Groups		Group (III)			Group (IV)		
Parameters	Range	Mean ±SE	P value	Range	Mean ±SE	P value	Normal range
ALT(SGOT)	91.1-94	92.38±2.3	>0.05	99.9-121	111±7.3	<0.0001	88.1-147
AST(SGPT)	40-42.6	41.5±0.9	>0.05	87-105	96.55±6.9	<0.0001	35-80
Urea	13-19	14.33±1.2	>0.05	33-43	36.67±3.8	<0.0001	5-29
Creatinine	0.55-0.62	0.58±0.1	>0.05	0.82-0.98	0.9±0.1	<0.0001	0.5-1

Table 3a and 3b: show clinical pathological effect in CBC in treated rats: P value >0.05: no significant results in compared to control, P value <0.0001: significant results in compared to control. ALT: Alanine aminotransferase, AST: aspartate aminotransferase.

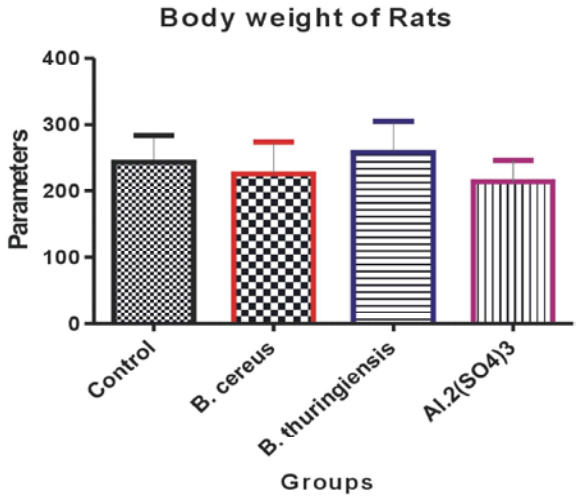


Fig. 1: Effect of extracellular bioflocculants on body weights of rats

From the above it seems clear that the (glycoproteins) of *Bacillus thuringiensis* has increased rats weight during the term of the experiment

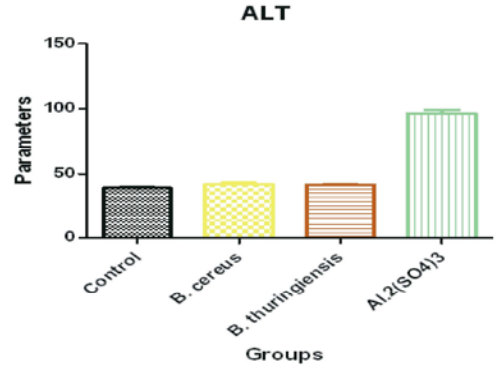


Fig. (a)

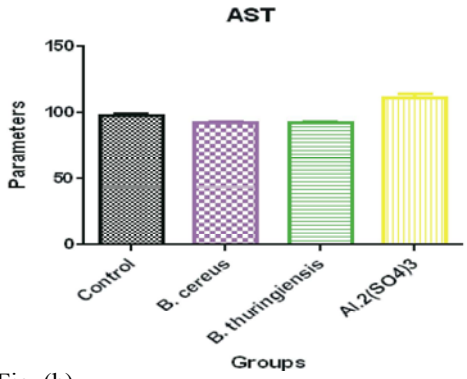


Fig. (b)

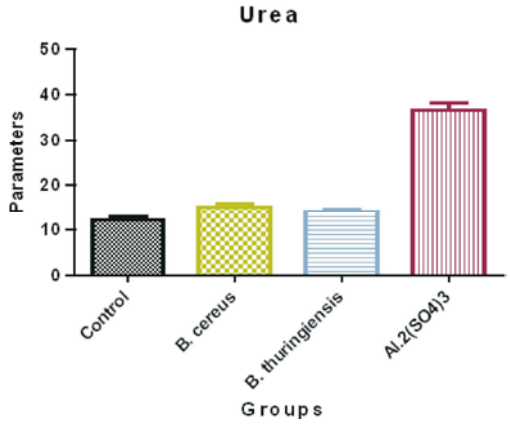


Fig. (c)

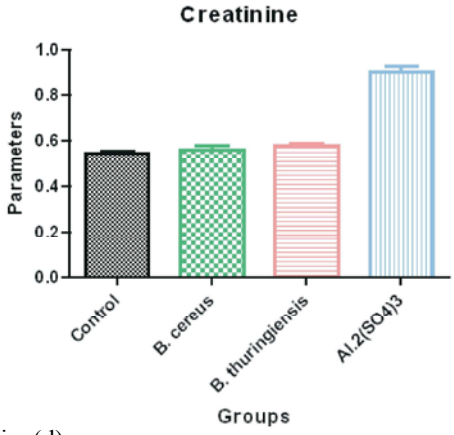


Fig. (d)

Fig. 2: Effect of extra-bioflocculants on serum liver enzyme activities and kidney function on rats

The data of these figures revealed to; no significant differences in liver enzymes ALT and AST and kidney function between control and group II and III that received a tested bio-flocculant material (glycoproteins) from *Bacillus cereus* and *Bacillus thuringiensis*, while Aluminum sulfate administered rats exhibited a highly significant excess in liver enzymes ALT and kidney function by time in compared with control

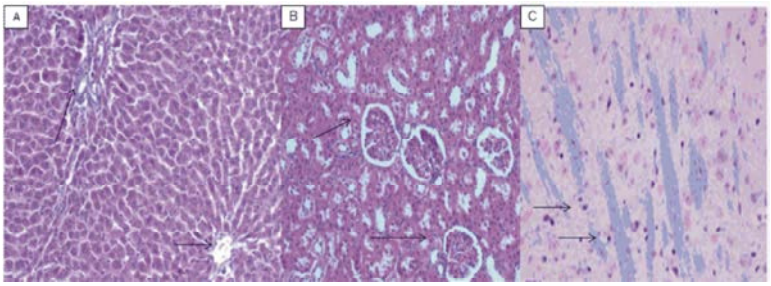


Fig. 3: Light micrograph of tissue from control rats. (6a): rat liver showing normal structure liver cells, central vein (→) and arrangement of hepatic cells with centrally placed nucleus (→). (6b) Control rat kidney with normal renal tubules (→) and golmeruli (→). 6(c) Rat control brain showing normal myelin display from rat brain (→ blue stained). X = 200

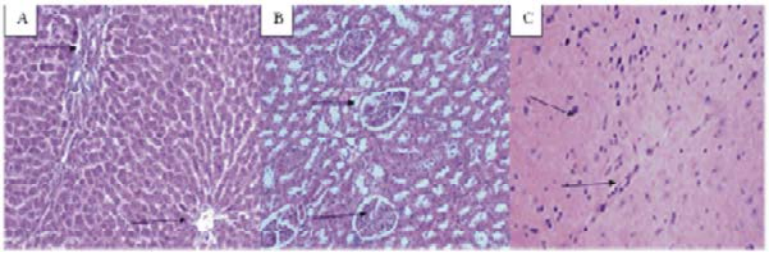


Fig. 4: Light micrograph from tissue of rat that recevied bioflocculants (glycoprotein's) of *Bacillus cereus*. (7a) nearly structure liver cells, central vein (→), arrangement of hepatic cells with centrally placed nucleus (→). (7b) kindney with nearly normal renal tubules (→) and glomeruli (→). (7c) brain showing nearly normal myelin display (→) blue stained). X = 200

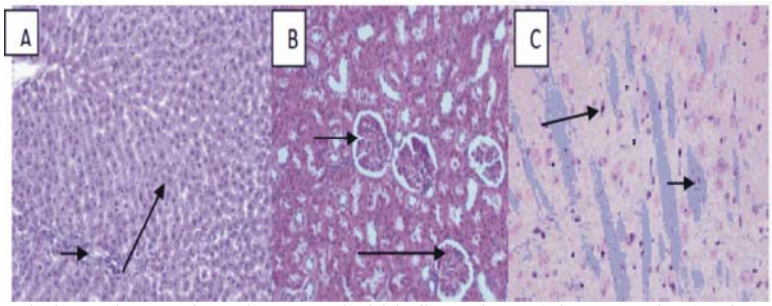


Fig. 5: Light micrograph from tissue of rat that recevied bioflocculants (glycoprotein's) of *Bacillus thuringiensis*. (8a) nearly structure liver cells, central vein (→), arrangement of hepatic cells with centrally placed nucleus (→). (8b) kindney with nearly normal renal tubules (→) and glomeruli (→). (8c) brain showing nearly normal myelin display (→) blue stained). X = 200

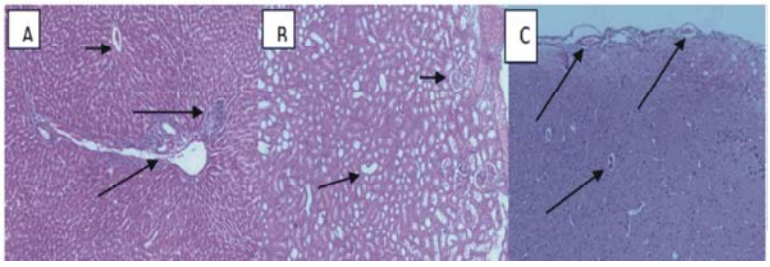


Fig. 6: Light micrograph fromtissue of rats that received $Al_2(SO_4)_3$. (9a) the liver show aggregation of moninuclear cells at portal area (→), congestion, slight vaculation in the cytoplasm of same hepatocytes, pyknosis of some nuclei of hepatocytes and arrangement of hepatic cells with centrally placed nucleus (→). (9b) kidney shows congestio, cellular cast and slight dilatation in collection tubules (→). (9c) the brain showing prevascular edema (→), vaculation and congestion in the blood vessels. X = 200

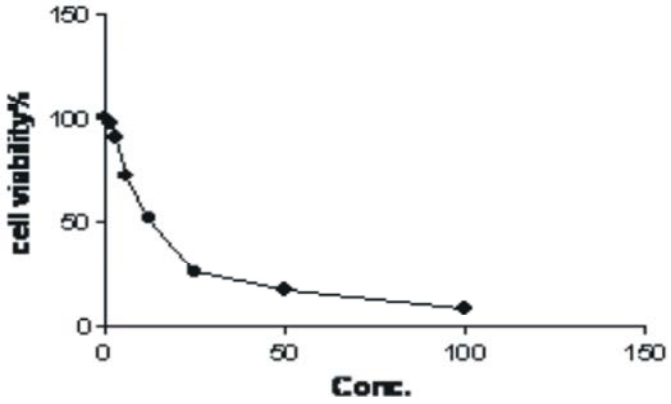


Fig. 7: Cytotoxic effects of different parasporal protein dilutions of *B. thuringiensis* on Ehrlich Ascites Carcinoma cells (EAC)
The figure shows cell viability of EAC cells at different concentrations/mg determined by O.D at 564nm after 24 hours incubation

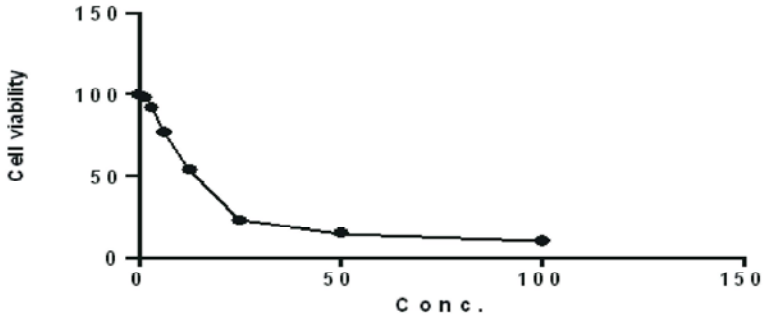


Fig. 8: Cytotoxic effects of different parasporal protein dilutions of *B. cereus* on Ehrlich Ascites Carcinoma cells (EAC). The figure shows cell viability of EAC cells at different concentrations/mg determined by O.D at 564nm after 24 hours incubation

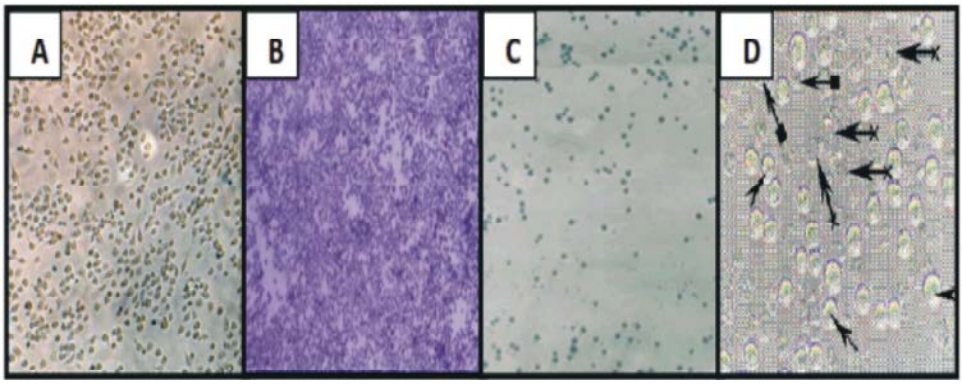


Fig. 9: Cytopathic effect of bacterial metabolites (parasporal proteins) cell extracts of *B. thuringiensis* and *B. cereus* on Ehrlich Ascites Carcinoma (EAC) cells. (A) Untreated cells are viable with normal morophology; dead cells stained with trepan blue, only dead cells take the blue color. (B) Untreated cells stained with crystal violet stain, viable cells take the stain while dead cells not stained. (C) Treated cells with proteins cell extracts of *B. thuringiensis* and *B. cereus* was affected stained with neutral red and show reduction in viabel cell number commpared to untreated cells (A & B). (D) Cells treated with proteins cell extracts of *B. thuringiensis* and *B. cereus* show cells with degenerative changes, loses of cell morphology and release of thier contents (←) which finally lead to death of cells after 24h of incubation. Untreated cells show overgtowth and complete distribution with normal cell morphology (A & B). X = 200

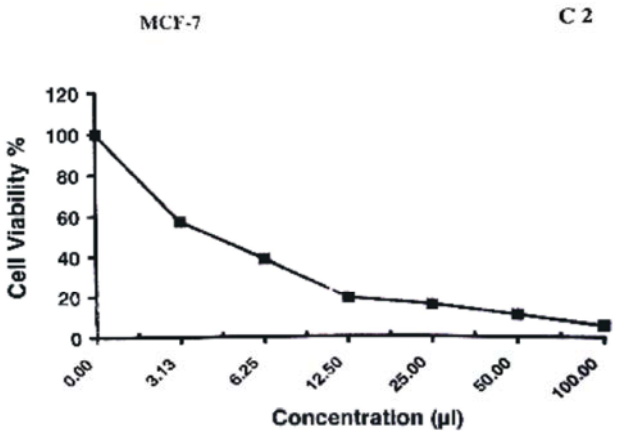


Fig. 10: Cytotoxic effects of different parasporal protein dilutions of *B. cereus* on Breast carcinoma cells (MCF-7)
From the results it's clear that: inhibitory activity against Breast carcinoma cells was detected under this experimental condition with LC50= 10.87mg/ml determined by O.D at 564nm after 24 hours incubation

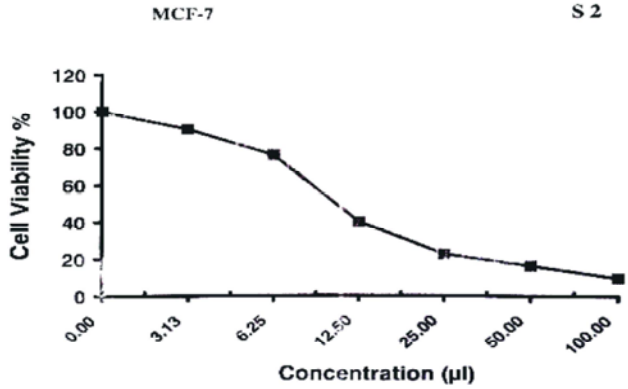
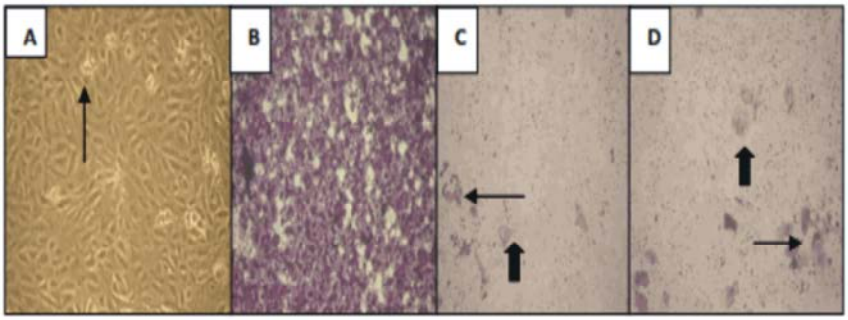


Fig. 11: Cytotoxic effects of different parasporal protein dilutions of *B. thuringiensis* on Breast carcinoma cells (MCF-7). The figure shows cell viability of MCF-7 cells at different concentrations/mg determined by O.D at 564nm after 24 hours incubation



(Figure 12): cytopathic effect of bacterial metabolites (parasporal proteins) cell extracts of B. thuringiensis and B. cereus on Breast carcinoma cells (MCF-7) cells. (A) Unstained untreated cells are viable with normal morphology (B) Untreated cells stained with crystal violet stain, viable cells take the stain while dead cells not stained, Untreated cells show overgrowth and complete distribution with normal cell morphology (A & B). (C & D) Cells treated with proteins cell extracts of *B. thuringiensis* and *B. cereus* and stained with crystal violet show cells with degenerative changes, loses of cell morphology(⬆) and release of their contents (→)which finally lead to death of cells after 24h of incubation. X= 200.

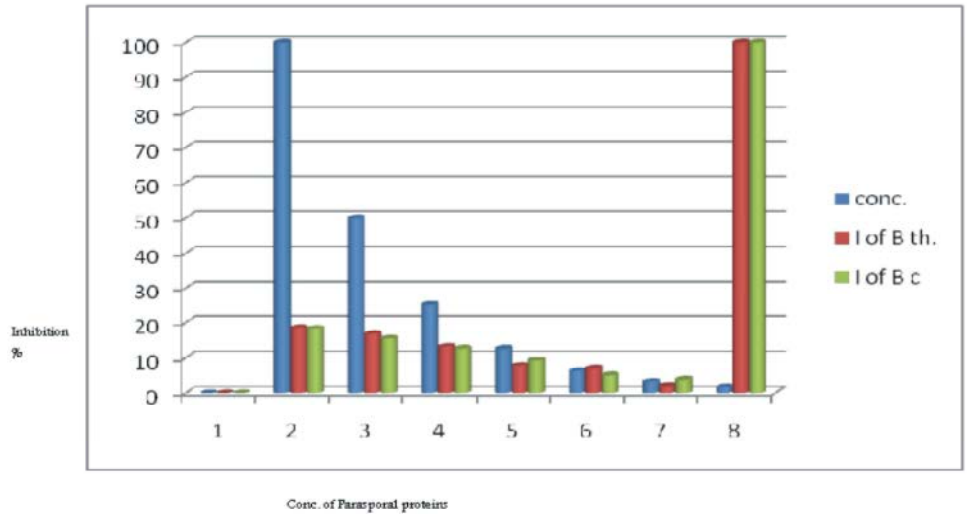


Fig. 13: Cytotoxic effect of different conc. of parasporal proteins of *B. cereus* and *B. thuringiensis* on Fibroblast cell line [(MRC-5) normal cells of human Lung] measured by O.D at 564 nm after 24 hours incubation

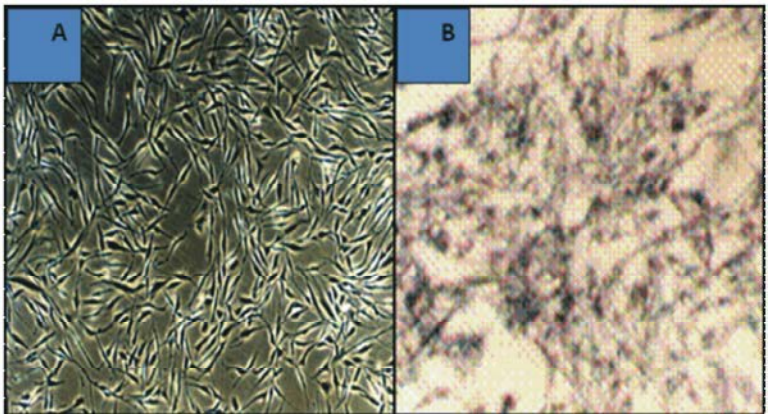


Fig. 14: Effect of Parasporal proteins of *B. cereus* and *B. thuringiensis* on Fibroplast cell line [(MRC-5) normal cells of human Lung]
(A) Untreated cells show complete distribution with normal cell morphology (spindle-shape). (B) Cells that treated with Parasporal proteins of *B. cereus* and *B. thuringiensis* show slightly effect with bacterial proteins X= 200

Histological Examination: Examination of liver sections of the control group revealed normal histological structure of the central vein and surrounding hepatocytes and there was no histopathological alteration observed. The kidney also shows normal histological structure of epithelial cells and normal renal tubules. Also, the brain of rats in control group show normal histological structure as shown in Figures 3a, 3b and 3c. Administration of bioflocculants (glycoproteins) of *Bacillus cereus and Bacillus thuringiensis* give the same results Figures 4a, 4b, 4c, 5a, 5b and 5c, respectively. On the other hand, the administration of $Al_2 (SO_4)_3$ exhibited pathological changes in liver, kidney and brain as shown in Figures 6a, 6b and 6c.

In vitro* of Anticancer Activities of Parasporal Proteins of *B. thuringiensis* and *B. cereus

Cytotoxicity Assay: *B. thuringiensis* and *B. cereus* were used for detection of antitumor activity on Ehrlich Ascites Carcinoma cells (EAC) by using trypane blue stain as shown in Table 4.

Cytocidal Effect of the Different Concentrations of *B. thuringiensis* and *B. cereus* Parasporal Proteins on the Viability of EAC & MCF-7 after 24h Incubation: In this study, crystal violet based cytotoxicity assay was used as it is a most sensitive and advanced technique used in this field for evaluation of anticancer drug *in vitro*. The total intracellular protein extract having

Table 4a: Effect of intra-extracellular extracts of *B. thuringiensis* and *B. cereus* on Ehrlich Ascites Carcinoma [EAC]

Bacterial organism	Extracellular extract	Intracellular extract
B. thuringiensis	-ve	+ve
B. cereus	-ve	+ve

From the results in table 4 it was clear that bacterial intracellular extract were + ve as shown from testing on EAC.

Table 4b: Effect of Parasporal proteins of *B. thuringiensis* and *B. cereus* on Ehrlich Ascites Carcinoma [EAC].

Sample conc. (µl)	Viability % of *B. thuringiensis*	Inhibitory %	Viability % of *B. cereus*	Inhibitory%	±SD
100	9.27	100%	10.7	100%	±1.26
50	18.3	89.5%	15.3	85.5%	±2.5
25	26.5	76.8%	23.5	72.8%	±2.44
12.5	52	57.2%	54	59.1%	±3.6
6.25	72.2	27%	77.2	32%	±4.25
3.125	90.6	7%	92.6	9%	±1.39
1.56	97.3	1.2%	98.4	3.6%	±0.0368
0	100	0	100	0	± 0.0435

From the results it's clear that: inhibitory activity against Ehrlich Ascites Carcinoma cells (EAC) was detected under these experimental condition with LC50=11.3µg/ml for *B. thuringiensis* & 9.5µg/ml for *B. cereus* respectively

Table 5: Effect of Parasporal proteins of *B. cereus* on Breast carcinoma cells [MCF7]

Sample conc. (µl)	Viability %	Inhibitory %	Standard Deviation
100	5.62	94.38	±0.94
50	10.97	89.03	±1.12
25	15.84	84.16	±1.43
12.5	19.28	80.72	±2.51
6.25	38.65	61.35	±3.73
3.125	57.46	42.54	± 6.22
1.56	79.13	20.87	±3.15
0	100.00	0	--

From the results it's clear that: inhibitory activity against Breast carcinoma cells was detected under these experimental condition with LC50=4.36µg/ml

Table 6: Effect of Parasporal proteins of *B. thuringiensis* on Breast carcinoma cells [MCF7]

Sample conc. (µl)	Viability %	Inhibitory %	Standard Deviation
100	9.76	90.24	±1.46
50	16.42	83.58	±0.98
25	22.58	77.42	±1.74
12.5	40.33	59.67	±3.51
6.25	76.96	23.04	±5.82
3.125	90.67	9.33	±1.71
1.56	98.45	1.55	±0.43
0	100.00	0	--

From the results it's clear that: inhibitory activity against Breast carcinoma cells was detected under these experimental condition with LC50= 10.87µg/ml.

Table 7: Effect of Parasporal proteins of *B. thuringiensis* and *B. cereus* on Fibroblast cell line [(MRC-5) normal cells of human Lung]

Sample conc. (µl)	Viability % of *B. thuringiensis*	Inhibitory %	Viability % of *B. cereus*	Inhibitory %↑	±SD
Untreated control	100%	0	100%	0	±1.2
100	81.5	18.5	81.7%	18.3	±2.15
50	83.2	16.8	84.5%	15.5	±2.45
25	86.8	13.2	87.46%	12.54	±3.63
12.5	92.1	7.9	90.68%	9.32	±4.21
6.25	93	7	94.9%	5.1	±1.3
3.125	98.1	1.92	96.3%	3.7	±0.038
1.56	100	100	100%	100	± 0.045

From the results it's clear that: inhibitory activity Fibroblast cell line [(MRC-5) normal cells of human Lung] was detected under this experimental condition 18.5 µg/ml for *B. thuringiensis* & 18.3 µg/ml for *B. cereus* respectively

a preferential cytotoxicity against tumor cell lines EAC, MCF7 at high concentration were 100%, 90.24% respectively for *B. thuringiensis* with lethal concentration 50 (LC50) of 10.87µg/ml. while was 100%, 94.38% respectively for *B. cereus* with lethal concentration 50 (LC50) of 4.36 µg/ml as shown in Tables 4, 5 and 6, Figures 7, 8, 9, 10, 11 and 12, while the toxicity of normal cells (MRC-5) was lower effect with 18.4% as shown in Table 7 and Figure 14.

DISCUSSION

Flocculation remains one of the most common and important processes in water treatment [1]. There are concerns regarding the use of inorganic metal salts in coagulation-flocculation process. Aluminum salts are by far the most widely used coagulants in water treatment due to its convenience and cost-effectiveness. However, several serious disadvantages of using Aluminum salts including Alzheimer's disease and similar health related problems associated with residual aluminum in treated waters have been identified [4]. To overcome these health concerns, scientists tried to find alternates for using of Aluminum salts in water coagulation. In recent years, health concerns were arising about coagulation-flocculation agents. Various epidemiological studies have suggested that Aluminum plays a pathogenic role in age-related changes and neurodegenerative diseases such as Alzheimer's disease [6, 7]. Besides the brain, Aluminum accumulates in kidney and liver in high doses, Aluminum was also shown to damage the kidneys, bones, heart, lungs and hematological system but unfortunately very little data exists for other organs [5]. One contemporary approach that had received attention was the probing of the bioactive substance isolated from natural sources. One of these natural sources is the using of bacterial metabolites as biofocculants [8]. Bioflocculants are kinds of extracellular biopolymers of macromolecular substances such as proteins, glycoproteins, polysaccharides and nucleic acids that mostly come from the natural secretions of microorganisms and cell lyses [9, 10]. Bioflocculants have recently attracted and received a considerable scientific and biotechnological attention, especially due to their biodegradability, non-toxicity and lack of secondary pollution [9, 21]. In our previous work, we have found that *Bacillus cereus* and *Bacillus thuringiensis* have extracellular bioflocculant (glycoproteins) that was used as a coagulant in raw water treatment instead of aluminum sulfate [2]. Both species are members of the *Bacillus cereus* group of bacteria. *Bacillus* genus includes a variety of medical and industrially important species. This genus encompasses a great diversity of bacterial species and has a ubiquitous distribution in the environment. Because of their resistant spores and the capacity of vegetative cells to secrete a wide variety of enzymes, this genus is important for the production of commercially important bio-molecules (insecticides, enzymes, antibiotics, antitumor, pesticides, etc.) [1]. Hence, it is essential to study the cytopathic effects of both of Aluminum salts and bioflocculants in biological system, which will give some idea about their toxicity. The liver, kidney and brain were selected to describe the histological changes after bioflocculant (glycoproteins) and Aluminum exposure. The liver via the portal vein is the first organ exposed to internally absorbed nutrients and other xenobiotics. The liver is composed of highly active metabolic tissue containing huge complement of detoxification machinery system [22]. This regarded with our histological and biochemical results on experimental animals that received aluminum sulfate as shown in Tables 1, 2 and 3, Figures 6a, 6b and 6c. Treatment with $Al_2 (SO_4)_3$ in the present study exhibited pathological changes on liver, kidney and brain. The liver show aggregation of mononuclear cells at portal area, congestion, slight vaculation in the cytoplasm of some hepatocytes, pyknosis of some nuclei of hepatocytes and arrangement of hepatic cells with centrally placed nucleus. Kidney shows congestion, cellular cast and slight dilatation in collection tubule. The brain show prevascular edema, vaculation and congestion in the blood vessels. This may indicate the direct effect of Aluminum sulfate on the walls of blood vessels, which leads to changes in the components of the walls, leading eventually to become the walls permeable liquid components of blood and this lead to infiltration around the blood vessels in this tissue [5]. Also, the direct effect of Aluminum sulfate on capillaries leads to the withdrawal of red blood cells through these tissues, particularly the liver and brain and this described the bleeding through these tissue, as well as, cellular changes that seen in the kidneys and liver included bulges and cellular edema described changes the solubility in the cells, may be attributed to direct effect of Aluminum sulfate on cellular compositions including organelles and cell membranes which lose optional permeability and leading to the entry and accumulation of fluid into the cells and the emergence of cytoplasmic bubbles that gather inside the liquid. The presumably that with continued experiment and exposure the tissues to Aluminum sulfate, the changes progress to necrosis due

to the cells lake the ability to resist the adverse effect of Aluminum on the organelles and membranes which leading to necrosis that characterized by nuclear chromatin broken and analyze of cytoplasmic mass of cells. Many studies showed that Aluminum accumulates in the body in the liver, brain and kidney cells by 50-75% of the amount of Aluminum accumulated in the body. These findings are consistent with other results from Aluminum experiments on tissues such as liver and kidney like Mahitha *et al.* [5]. The groups of rats that received bioflocculant (glycoproteins) from *B. thuringiensis* and *B. cereus* were not affected by these materials and show no significant results in compared to control animals, as shown in this study. It is interesting from the results that the (glycoproteins) of *Bacillus thuringiensis* has increased Rats weight during the term of the experiment, suggesting that the (glycoproteins) is not toxic to organs of the body and may also evidenced by that this glycoprotein may be useful in other areas, for some people who suffer from thin but it necessary to hold many of the experiments in the future.

On the other hand, the clinical trials for treatment of cancer using bacterial metabolites and their toxins are run in many significant approaches including bacteria and their toxins as immunogen, immune stimulants like *Salmonella* spp. [20] and *Listeria* toxins [21] or as adjuvant for the chemotherapeutic agents like *Bacillus calmette guerin* (BCG) [22]. Others used bacterial toxins as preferential cytotoxic agent for cancer cells like using diphtheria toxin for treatment of prostate cancer cells [23] and using *Bacillus thuringiensis dakota* δ-endotoxins for treatment of Ehrlich Ascites Carcinoma in mice [24]. Historically, it has been believed for many years that *B. thuringiensis* has acquired insecticidal activity in the course of co-evolution with insects through a host-parasite relationship. This hypothesis is attractive for many investigators; however, circumstantial evidence leads to another idea that *B. thuringiensis*, as a species, is merely an environmental saprophyte but not an obligate pathogen of insects. This is supported by the fact that in natural environments *B. thuringiensis* isolates with non-insecticidal Cry proteins outnumber the insecticidal ones [20, 21]. It is noteworthy that the non-insecticidal isolates often account for >90% of the natural populations from soils [22] and phylloplanes [25, 26]. Thus, the important question that naturally arises is whether Cry proteins synthesized in non-insecticidal *B. thuringiensis* have any biological activity which is as yet undiscovered [27]. Based on the above historical background, an extensive screening of *B. thuringiensis* Cry proteins that have novel biological activities other than insect toxicity was commenced in 1996. This has led to the discovery of the unique proteins that target human cancer cells and a human-pathogenic protozoan [28]. Our results observed that bacterial metabolites (parasporal proteins) cell extracts of *B. thuringiensis* and *B. cereus* have a cytocidal effects on breast carcinoma cells (MCF-7) cells and on Ehrlich Ascites Carcinoma (EAC) cells and its EC50 was 10.87µg/ml and 5.5 µg/ml µg/ml respectively. Regarding to Figures 9 and 12 it is definitely clear that there is a great variation in the degree of tumor cells susceptibility as EAC cells showed the highest susceptibility to parasporal proteins with EC50 5.5µg/ml. The normal cells MRC-5 did not showed any significant cytotoxicity. The above mentioned observations provide strong evidence that bacterial metabolites (parasporal proteins) cell extracts of *B. thuringiensis* and *B. cereus* are highly selective for the tested cancer cells. The cytopathological changes, induced by parasporal proteins, in tested cancer cell lines (EAC and MCF-7) were characterized by marked cell ballooning accompanied with cytoplasmic hydropic degeneration and cytoplasmic vaculation followed by nuclear kariolysis and disruption of the cytoplasmic membrane then ended by drastic cell lysis with release of cellular contents to outside. In contrast, these activated proteins didn't induce any cytopathological changes in MRC-5 normal cells at concentrations up to six times the LC_{50} of MCF-7 cells Figure 14. In respect of the cytopathological change by parasporal proteins of *B. thuringiensis* and *B. cereus* resemble the cytopathological changes that induced by *Bacillus thuringiensis* parasporal inclusion proteins (δ-endotoxin) in the tumor cells [24]. So, we suggest that their mode of action is mainly on the cell membrane of the tumor cells.

CONCLUSION

The bioflocculant extracted from extracellular product (carbohydrate + protein) of these bacteria can be used as anticoagulant [instead of $Al_2(SO_4)_3$] in the field of water and wastewater treatment in the future due to their biodegradability, non-toxicity, benign nature and lack of secondary pollution as confirme in the study. Also, the (carbohydrate + protein) of *Bacillus thuringiensis* that had increased Rats weight during the term of the experiment may be useful in other areas, for some people who suffer from thin but it necessary to hold many of the experiments in the future. On the other hand, The identification of these activated protein may be introduced as a novel save natural anticancer agent

derived from bacterial source and points to the importance of exploring these raw products and opens new horizons in the field of development of pharmacological tools in cancer therapy.

It has been recommended to verify if these bioflocculants of *B. thuringiensis* and *B. cereus* will keep their potent safety power in bearing animals in the future or not and also if parasporal proteins cell extracts of *B. thuringiensis* and *B. cereus* will keep their potent anticancer power in cancer bearing animals.

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