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Selective Anti-malignancy Activity of *Paracoccus alcaliphilus* Alkaline Proteins (PAAP) Against Mammalian Cell Lines *in vitro*

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Abstract: Anticancer agent derived from *Paracoccus alcaliphilus* (PAAP) was conducted. Three cancer cell lines were employed in this study [Hepatocellular Carcinoma (HEP-G2), Central nervous system (U251), Ehrlich Ascitis Carcinoma (EAC) and one normal cell lines as control [Fibroblast cells of human lung (MRC5)]. The *P. alcaliphilus* alkaline protein (PAAP) showed *in vitro* preferential cytotoxicity to human and animal cancer cell line. Alkaline soluble proteins revealed dose dependent cytotoxicity against two tumor cell lines (EAC and HEP-G2) in a corresponding lethal concentration 50 (LC_{50} were 5.5 and 21 μg ml^{-1}), respectively. While CNS (U251) did not show any significant cytotoxicity by the alkaline protein extract. The alkaline protein selectivity to malignant cell lines was clear as the normal cell line (MRC-5) did not affect even by the highest concentrations. The cytopathic effect induced by PAAP was characterized by remarkable kariolysis of the nucleus, cell ballooning, loss of cell membrane integrity ended with complete cell lysis. The electrophoretic pattern using Native-PAGE for the alkaline protein extract of *P. alcaliphilus* revealed that it consists of single protein fraction, stained clearly by sensitive silver stain, which is fractionated later via SDS-PAGE into three subunits with M. Wt. (25, 20 and 19 kDa, approximately). It is tempting from the data presented in the paper that *P. alcaliphilus* alkaline protein has a significant and preferential cancer cell-killing activities that may Pave the way in the near future to cancer control using biotherapy.

Key words: *Paracoccus alcaliphilus* · HEPG2 · U251 · EAC · MRC-5 · Cytotocicity

INTRODUCTION

The use of chemotherapy and/or radiotherapy in the treatment of cancer was developed during the last century and showed a limited success when compared with the dangerous side effects that occurred either during or after treatment courses [1]. In addition to the uncontrollable cancer, metastasis that commonly occurred in the majority of treated cases and caused dramatic end of their lives [1]. Consequently, interest has been paid to using alternative strategies for cancer treatment [2]. It is little, if any, is known about the production of anti-cancer agent from Halobacteria especially those belonging to Genus *Paracoccus*.

The only researches present in this field is about taking the advantage of remarkable similarity at the molecular biology level between Halobacteria and eukaryotic cells so it could be used in microbial prescreening for new anti-cancer agents [3]. It was assumed also that due to the similarity demonstration between *c-myc* gene products of cancer patients and a 84kDa protein isolated from cell extracts of halobacterium balobium [4] halobacteria could be used in diagnosis of certain cancer and also for devised to detect the levels of antibodies in patient's serum, which is clinically relevant [5].

Paracoccus are among the most metabolically versatile bacteria. They are chemo-organo-heterotrophs

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or facultative chemo-litoauto-trophs as it can grow in anaerobic conditions (nitrate respiration) as *Paracoccus* can play an important role in the cycling of elements in the environment because of their versatile metabolism [6]. *Paracoccus alcaliphilus* is methanol-utilizing bacteria, facultative methylotrophic, non-spore forming, Gram-negative, non-motile, coccoid or short rod-shaped organism with a Q-10 ubiquinone system, a cellular fatty acid composition consisting of a large amount of C18:1 acid (type B) and a hydroxyl fatty acid composition consisting of a large amount of 3-OH C10:0 acid and 3-OH C14:0 acid. However till now there is now publication record about the using of *Paracoccus alcaliphilus* cellular metabolites for controlling of malignancy *in vitro*. Therefore, this study aims to investigate the anticancer activity of *P. alcaliphilus* proteinatious metabolites that belonging to haloalkalophilic microorganisms, *in vitro* against the frequently known cancer cell lines and their ability to discriminate between normal and cancer cells.

MATERIALS AND METHODS

Bacterial Strain: *P. alcaliphilus* used in this study was isolated from Qaroun calcareous clay loam soil. It was grown at 37°C for 48h on Sato agar medium [glucose (10 g), peptone (5g), yeast extracts (5g), NaCl (150 g), K_2HPO_4(1 g), Na_2CO_3 (10 g, $MgSO_4.7H_2O$ (0.2 g), agar (15 g) and distilled water (1000 ml)] (pH 10.5) [7].

Identification of *P. alcaliphilus*: Direct microscopical examination of the isolates was carried out by using routine stain (Gram's stain) according to Cruickshank *et al*. [8]. Scanning Electron microscope examination was carried out according to Yamamoto *et al*. [9] were bacterial mixture was suspended into a drops of bi-distilled water on an aluminum disks and then leaved to dried on air. The samples were coated by gold and then were examined using a scanning electron microscope (JEOL model JSM-S1).

The physiological and biochemical characters of the *P. alcaliphilus* were studied according to the principals and the recommended methods described by Urakami *et al*. [10]. The physiological and biochemical characterization included the following tests: catalase, oxidase tests, nitrate reductions, indol, maximum and minimum growth temperature, growth in sodium chloride, growth at pH 10.5-12.5, sugars fermentation tests, H_2S production, starch, casein, tween 20, tween 40, tween 80 hydrolysis, gelatin liquefaction and urea utilization, nitrogens sources requirement, antibiotics sensitivity.

Solubilization, Purification and Characterization of *P. alcaliphilus* Proteins: Bacterial cultures were harvested by centrifugation at 10,000 g for 30 min. at 4°C and washed three times in 1M NaCl containing 5% lactose. Lyophilized bacteria were grinded well using mortar for 4 h and re-suspended in Phosphate buffer with different pH (7.2, 10 and 4) prepared according to Gomori, [11] as a pH gradient for dissolving intracellular proteins. The dissolved proteins were precipitated with 90% saturated ammonium sulfate solution. The resulted precipitates were dialyzed against 2X distilled water each is 2 liter overnight at 4°C. The resulted soluble proteins were separated by centrifugation at 10,000 g for 30 min at 4°C. The resulted soluble proteins were purified from the small protein peptides by using Vivaspine concentrator with cut off 10 kDa. The purified alkaline, neutral and acid soluble protein fractions concentration and recovery percentages were monitored by using method of Bradford [12] with using bovine serum albumin as standard protein.

Electrophoresis Analysis of Cytotoxic Alkaline Soluble Protein Fraction (PAAP): Native and SDS-PAGE was performed as described by Laemmilli, [13] using 4% stacking and 12% resolving gels. After electrophoresis, the gels were stained with 0.1% (W/V) silver nitrate according to Danielm and Stnartj [14]. The size of the protein mass was determined by using molecular mass standards Sigma@. (Created by Lablmage Version 2.7.0. by Kapelan Gmb H., 1999-2001).

Tissue Culture Cell Lines: Hepatocellular Carcinoma cells (HEP-G2), central nervous system cells (U251), Ehrlich Ascites Carcinoma cells (EAC) were supplied kindly from National Cancer Institute (NCI), Egypt, Fibroblast human lung (MRC5) was obtained from VACSERA, Egypt. All cell lines were maintained and propagate according to the instructions supplied from cell culture manufacturer.

Short-Term Viability Assays: In this study, Short-term viability-assay (trypane blue) was carried out according to Ian Freshney[15] to measure the proportion of viable cells following a potentially traumatic procedure such as primary disaggregating, cell separation, or freezing and thawing.

Long-Term Survival- Assay (MTT-Based Cytotoxicity Assay): In this study, MTT-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* according to Mizuki *et al*. [16].

Cytotoxicity Assay: A cell suspension of 100 µl, 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 [15]. Then each well received 50 µl of the fresh Dulbicos Modified Eagles Media (DMEM) and 50 µl of alkaline soluble proteins fraction solution in concentration ranging from 0.047 to 66.6 µg ml^{-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 cytopathic effects (CPE) at adequate intervals for 24 h under inverted microscope.

Cytopathic Effect: The degree of CPE was graded on the bases of the proportion-damaged cells in about 100 cells as follow: -, <5%: +, 5% to 10%; +, 10% to 30%; ++, 30% to<60%; +++, 60% to 90%; ++++,> 90%.as described by Mizuki *et al*. [16]. For assessment of the level of cytotoxicity, a cell proliferation test using MTT assay Sigma@ [17] was done 24h post-inoculation of the tested proteins. The average absorbance values in buffer inoculated negative control was used as blank value. The arbitrary unit was determined on the bases of the relative value absorbance (optical density at 564 nm) to the blank according to Heiss *et al*. [17]. The 50% lethal concentration (LC_{50}) values were deduced from the dose-response curve using log-probit program.

RESULTS

Light Microscopically Examination and Colony Character of *P. alcaliphilus*: Direct microscopical examination of *P. alcaliphilus* with Gram stain revealed that it is a Gram negative Coccobacili, non-spore, the colony of *P. alcaliphilus* was circular, convex, smooth entire edge, opaque, with different degrees of yellow color. The colony size was 2-3 mm diameter.

Electron Microscopically Examination of *P. alcaliphilus*: Scanning electron microscopy of *P. alcaliphilus* illustrated that it is a short rods and/or coccobacilli with rounded edges occurred singly, in pair or in irregular clumps (Fig. 1). The typical measurement is approximately 0.8 x 0.6 to 0.8 x 1.8 µm.

Table 1: The protein recovery during the process of purification of *P. alcaliphilus*

Fraction type	Protein Conc. In Microgram	Recovery (%)
1 gm lyophilized bacterial cells	-----	-----
Proteins soluble in neutral pH (7.2)	64.43	19.7
Proteins soluble in alkaline pH (10)(PAAP)	263.55	80.4
Proteins soluble in acidic pH (4)	Nil	Nil

Fig. 1: The colony of *P. alcaliphilus* cultivated on *Sato* agar medium at 37°C for 48 h

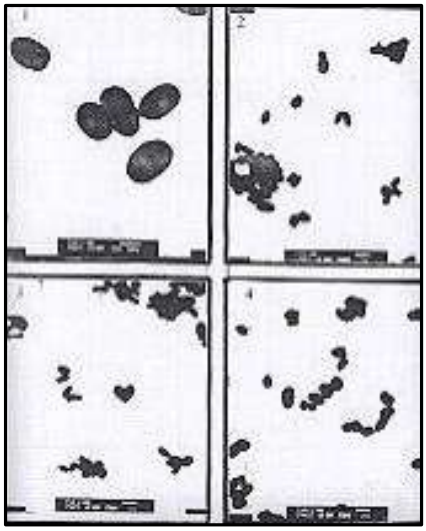

Fig. 2: Electron micrographs of the negatively stained cells of *P. alcaliphilus* at early stages and stationary phase.
1.18 h old culture (Bar 500 µm)
2.48 h old culture (Bar 1 µm)
3.48 h old culture (Bar 1 µm)
4.72 h old culture (Bar 1 µm)

Physiological and Biochemical Properties of *P. alcaliphilus*: The morphological, physiological and biochemical tests results were as follow: aerobic

growth +ve, growth at pH 6 +ve, growth at temperature (20°C, 30°C, 40°C, 50°C, 55°C) +ve, temperature tolerance at 70°C -ve, sodium chloride tolerance (1%, 3%, 10%, 15%, 20%, 25%) +ve, catalase test +ve, Sugars fermentation (glucose, mannose, starch, fructose) +ve, sucrose -ve, oxidase test -ve, gelatin liquefaction +ve, urease test +ve, nitrate reduction +ve. All recorded characters were identical to that of *P. alcaliphilus* in all confirmatory tests.

Purification and Solubilization of *P. alcaliphilus* proteins According to Their pH Solubility: The dissolved protein concentration and recovery percentage were measured using Bradford method [12], the total soluble protein for 1 gm of lyophilized bacterial cell was 327.98 µg/g. The highest protein concentration and recovery percentage was obtained by using phosphate buffer saline (pH10) for the alkaline protein (263.55 µg/g dry Wt. of bacterial cells represent 80.4% recovery percentage) followed by phosphate buffer saline (pH7.2) for the neutral protein, (64.43 µg/g dry wt. of bacterial cells represent 19.7% recovery percentage) and followed by using phosphate buffer saline pH 4 for acidic protein (nil) (Table 1). The final alkaline protein recovered from *P. alcaliphilus* was representing 80.4% of the total extracted bacterial proteins.

Native and SDS-PAGE of Purified Protein: Alkaline proteins of *P. alcaliphilus* was analyzed by non-denaturing (Native-PAGE) and denaturing (SDS-PAGE). Native-PAGE show that the protein is separated to only one intact protein band (Fig. 3) and SDS-PAGE show that this intact protein molecule was sub-fractionated to three subunits with molecular weights 25, 20 and 19kDa approximately, (Fig. 4, lane B).

Dose Response Cytotoxicity: The total intracellular protein extract having a preferential cytotoxicity against tumor cell line (EAC, HEP-G2 and U 251) while neither the normal cells (MRC5) nor the negative control cell lines were affected (Fig. 5). *Paracoccus alcaliphilus* alkaline soluble protein (PAAP) shows a highly cytotoxicity for EAC and HEP-G2 cells (Fig. 6), as their LC_{50} were (21 and 5.5 µg ml^{-1}, respectively). On the other hand it did not induce any significant cytocidal activity against U251 and normal control cell line (MRC-5).

Cytopathic Effect: It is clear that the cytopathic effect (CPE) was more prominent against EAC (21 µg ml^{-1}) then for HEP-G cells (5.5 µg ml^{-1}). The cytopathological changes, induced by the alkaline soluble proteins in both

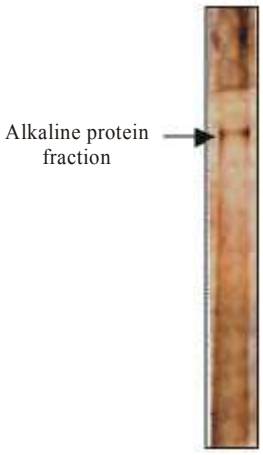

Fig. 3: Native-PAGE of *P. alcaliphilus* alkaline soluble protein (PAAP) stained with silver stain and shows only one intact protein fraction

EAC and HEP-G2 cells were characterized by marked cell ballooning with nuclear kariolysis and cytoplasmic vaculation followed by cell membrane disruption and ended with drastic cell lyses (Fig. 7B and D) while, the Central nervous system cancer cells (U251) did not show any cytopathological changes (Fig. 7 E and F). Normal control cells MRC-5 cells did not affected by the alkaline soluble proteins even in high concentrations when compared with the untreated cells (Fig. 7G and H).

DISCUSSION

Spontaneous tumor regression has followed bacterial, fungal, viral and protozoal infections. This phenomenon inspired the development of numerous rudimentary cancer immunotherapies, with the history spanning thousands of years. Coley took advantage of this natural phenomenon, developing a killed bacterial vaccine for cancer in the late 1800s. He observed that inducing a fever was crucial for tumor regression [18]. The clinical trials for treatment of cancer using bacterial metabolites and their toxins are run in many significant approaches including: using bacteria and their toxins as immunogen, immune stimulants like *Salmonella spp.* [19] and *Listeria* toxins [20] or as adjuvant for the chemotherapeutic agents like *Bacillus calmette guerin* (BCG) [21] or even as tumor-selective protein delivery vectors [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]. Another group

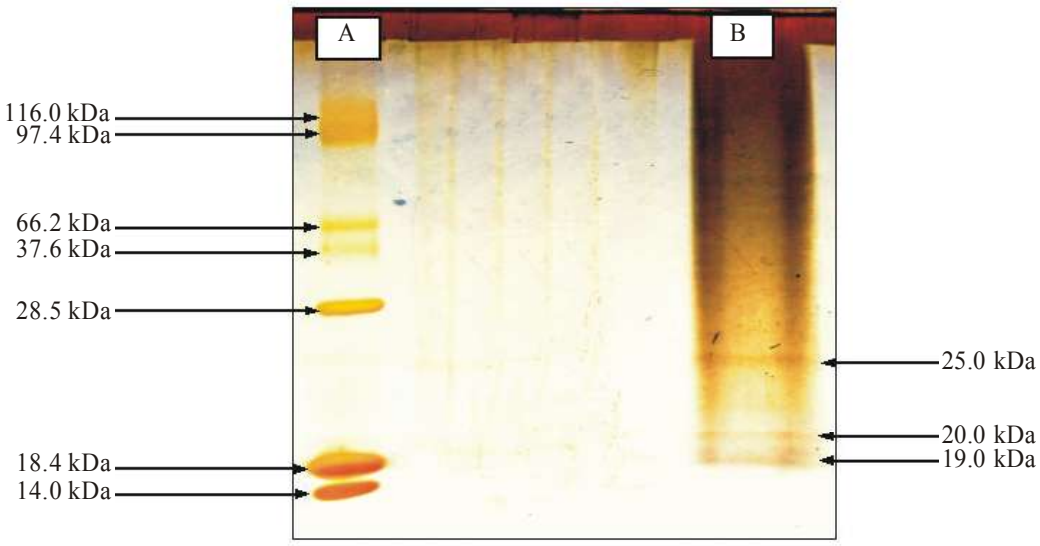

Fig. 4: 12% SDS - PAGE pattern of *P. alcaliphilus* alkaline soluble protein (PAAP). Lane (A) protein markers (Glactosidase 116 kDa, Phosphorylase-b 97.4 kDa, Bovine serum albumin 66.2 kDa, Alcohol dehydrogenase 37.6 kDa, Carbonic anhyrase 28.5 kDa, Myoglobin 18.4 kDa, Lysozyme 14.0 kDa, respectively. Lane (B) shows alkaline soluble protein with three subunit fractions of molecular weights (25, 20, and 19 KDa approximately)

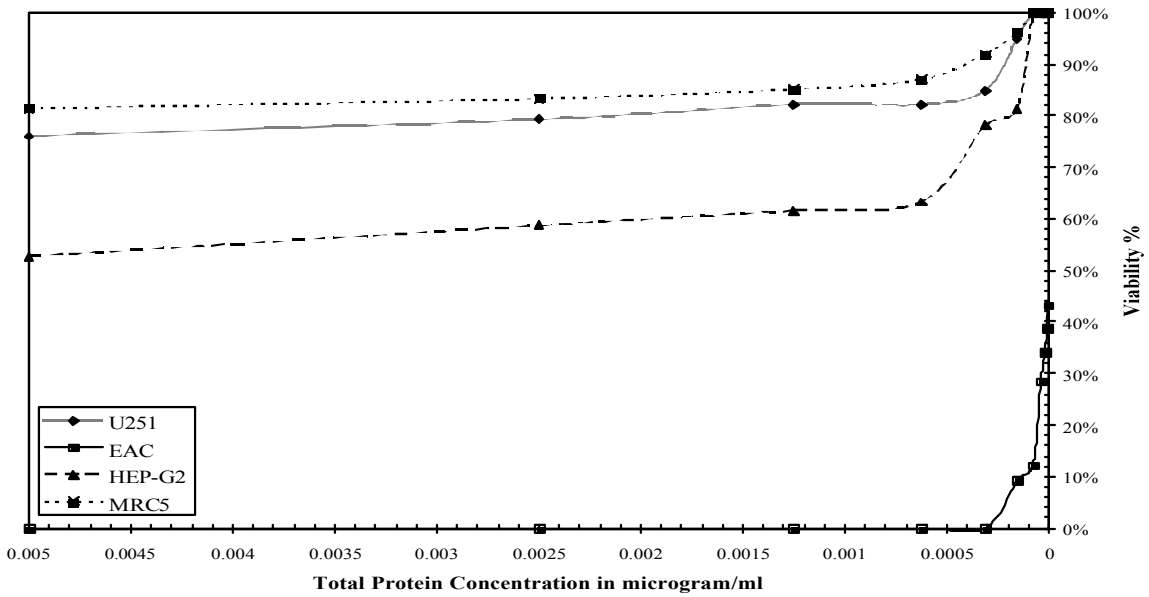

Fig. 5: Effect of *P. alcaliphilus* cell extract on normal cell line (MRC5) and cancer cell lines (U251, EAC and HEP-G2)

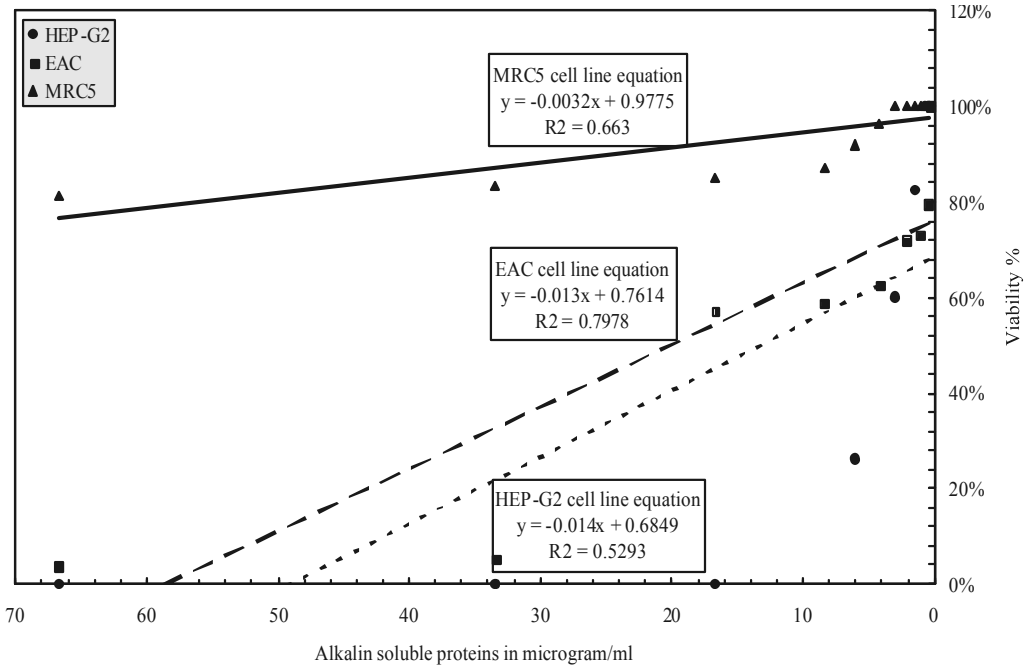

Fig. 6: Effect of *P. alcaliphilus* alkalin protein (PAAP) cell extract on HEP-G2, EAC and normal cell lines

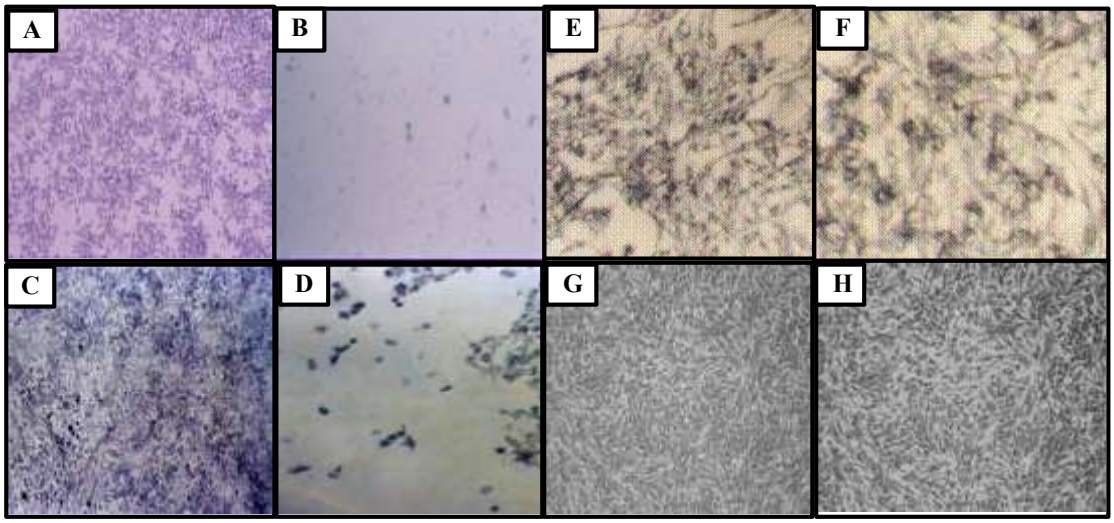

Fig. 7: Cytopathic effect of PAAP against HEP-G2, EAC and U251 (Fig. 7A&B, 7C&D, 7E&F, respectively). MRC-5 normal cells (Fig. 7G&H) using inverted microscopy 24h post treatment with magnification power 400X.

of researchers were used bacterial toxins for immunemodualtion and preferential cytotoxicity for cancer cells like *Pseudomonas* exotoxin [25]. The anticancer agent used in this research is intracellular protein in nature and this is new as most of previously interested authors in cancer biotherapy focused on bacterial toxins of Indol derivative [26] or Lipopolysaccharides (like those extracted from *Streptococcus pyogenes*) [27]. few of them focused in proteinatious endotoxins or metabolites like those use *Bacillus thuringiensis* δ-endotoxins [24].Regarding to the dose-response curve of *Paraoccus alcaliphilus* alkaline protein (PAAP) on EAC and HEP-G2 cells. The cytocidal activity of PAAP was in dose dependant manner with the cancer cells and its LC_{50} was 5.5 and 21 μg ml^{-1} (Fig. 5, 6) while CNS (U251) cancer cells were nearly insensitive to PAAP (Fig. 5,6). Regarding to Fig. 5 and 6 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 PAAP with EC_{50} 5.5 μg ml^{-1} followed by HEP-G2, However, these proteins only induce 18.4% cytotoxicity with the highest concentration used (131.175 μg ml^{-1}) on CNS cancer cells (U251). The normal cells MRC-5 did not showed any significant cytotoxicity. The above mentioned observations provide strong evidence that PAAP is highly selective for the tested cancer cells.

The cytopathological changes, induced by PAAP, in tested cancer cell lines (EAC and HEP-G2) 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 U251 nor MRC-5 normal cells at concentrations up to six times the LC_{50} of HEP-G2 cells (21 μg ml^{-1}) (Fig. 5 and 6). In respect of the cytopathological change reported in this study so far, resemble the cytopathological changes that induced by *Bacillus thuringiensis* parasporal inclusion proteins (δ-endotoxin) in the tumor cells [9]. So, we suggests that their mode of action is mainly on the cell membrane of the tumor cells. However, the elucidation of the exact site of action of these proteins on the cell membrane of the tumor cells needs further investigation.

It is important to verify if these PAAP proteins will keep their potent anticancer power in cancer bearing animals or not? This critical question is of author's interest and experiments are currently underway to answer this unsolved query.

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