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APOPTOTIC AND NECROTIC EFFECTS OF CHITOSAN NANOPARTICLES LOADED WITH THE HONEYBEE, *APIS MELLIFERA* VENOM ON DIFFERENT CANCER CELL LINES

By

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Abstract

Nanoparticles encapsulated with natural venoms have a considerable attention to improve illnesses therapies by overcoming toxic effects secreted by different pathogens. The present study explored conjugation of chitosan extracted from cuticle of naturally died honeybees nanoparticles (B-Cs-NPs) with the honeybee, *Apis mellifera* venom (BV). Detected related toxicity and anticancer potential of BV and BV loaded NPs- against Colon (CaCO-2), Larynx (HEp-2) and breast (MCF-7) cancer cell lines. The Inotropic gelation of chitosan (CS) with sodium tripolyphosphate (TPP) was the method used to prepare nanoparticles. Cytotoxic effects of BV and BV loaded NPs nanoparticles against the three cancer cell lines studied, then they were assessed using MTT stain assay. Data recorded revealed that combination of bee venom with bee chitosan nanoparticles enhanced the inhibitory effect against cancer cells studied when compared with bee venom alone. Also, it was cleared that by treating CaCO-2, HEp-2 & MCF-7 cell lines with IC_{50} concentrations of BV alone and BV loaded chitosan nanoparticles, this induced apoptotic features as cell shrinking, peripheral condensation of chromatin, apoptotic bodies in late stage, nucleoli fragmentation, membrane blebbing, shrunken nucleus with some necrotic features. Control cells showed normal pathway of mitotic divisions of obvious cell membrane and nucleus when stained by (H&E) and by visualization using inverted microscope.

Keywords: bee venom, chitosan nanoparticles, cytotoxicity, anticancer, cell lines

Introduction

The Hymenoptera are the third largest order of insects, comprising the sawflies, wasps, bees and ants. Stinging insects and the medical importance associated with their venoms are complex topics, and presentation of information pertaining to them requires the use of technical terms (Abdel-Rahman *et al*, 2015). Honeybee, *Apis mellifera* venom (BV) is composed of a variety of peptides, including melittin (a major component comprises approximately 50% of dry venom), adolapin, apamin, and mast cell degranulating (MCD) peptide (Park *et al*, 2004). In oriental medicine, bee venom not only used for treating rheumatoid arthritis, inflammation and pain (Son *et al*, 2007; Park *et al*, 2007), but it has anti-cancer potentials (Liu *et al*, 2002). Cancer is considered as a comprehensive predominant ailment that causes death in developing nations (Jemal *et al*, 2011; Calderon *et al*, 2014).The capability of many medications is often restricted by their ability to reach the target site of therapeutic action. In most cases, only a little quantity of applied dose reaches the targeted site, while the rest of the drug distributes throughout other organs of the body in accordance with its physicochemical and biochemical properties. So, the development of a drug delivery system that utilizes the pharmaceutical action of a medication while reducing its side effects *in vivo* is a difficult job. Recently, nanotechnology gained a wide attention of applications in the pharmaceutical manufacturing (Gupta, 2006). Because of the progress in nanotechnology, it is possible to produce drug encapsulated or entrapped nanoparticles to be used in an assortment of featuring designs (Jain, 2008). Polymers that are of chemical or natural

origin were used to prepare microspheres (nanoparticles) (Tiyaboonchai, 2003). But, among different polymers, chitosan has considered in pharmaceutical applications (Shahbazi *et al*, 2008). Also, it enhanced the penetration by opening tight epithelial junctions; used to deliver of both vaccines and proteins (Van der Lubben *et al*, 2001a,b). Nanoparticles prepared from chitosan have been widely assessed for the delivering of polypeptides such as insulin (Avadi *et al*, 2010), tetanus toxoid (Vila *et al*, 2004), diphtheria toxoid (Mokarram and Alonso, 2006), snake venom (Sarei *et al*, 2013), and proteins (Xu and Du, 2003).

The aim of the current work was to compare between the anticancer effect of BV and BV nanocapsulated chitosan of the honeybee, *Apis mellifera*.

Materials and Methods

Raw materials: Bodies of normally died bees, *Apis mellifera* (Hymenoptera: Apidae) were gathered from various apiaries at spring and utilized as a crude material.

Extraction of chitin and chitosan: Died bees were dried at 60ºC for 7 days and then subjected to successive five steps of treatment (Marei *et al*, 2016). (Fig. 1)

Preparation of bee chitosan Nanoparticles (B-CS-NPs) and Bee venom loaded chitosan nanoparticles (BV loaded CS-NPs): Bee Chitosan Nanoparticles were prepared by ionic gelation of bee Chitosan (B-CS) with trisodium polyphosphate (TPP). For Empty nanoparticles, 11ml of 0.33mg/ml of TPP were dropwisely added during stirring at 1000 rpm for 75 minutes, to 0.2gm of B-CS that was dissolved in 1% acetic acid. For bee venom loaded nanoparticles (BV loaded CS-NPs), 20, 50, 100, 200, 400 & 600µg/ml of bee venom was added to chitosan solution just before adding TPP and at strong acidic pH (Marei *et al*, 2016). Both empty and loaded NPs were separated by centrifugation at 14000 rpm for an hour, freeze dried at -40°C & 0.05 mbar then stored at 4°C±2°C. Weight of both loaded and empty NPs was recorded.

Cell culture and *in vitro* cytotoxicity assay: Colon cancer (CaCO-2), larynx cancer (HEp-2) and breast cancer (MCF-7) cell lines were grown in RPMI- 1640 medium supplemented with 10% fetal bovine serum (FBS), 1mM sodium pyruvate, 2mM L-glutamine & antibiotics (penicillin 100 IU/ml, streptomycin 100µg/ml). The cell cultures were grown at 37°C under 95% a humidified air and 5% CO2.The MTT assay was performed to assess the cytotoxicity of BV and BV loaded chitosan nanoparticles against cancer cell lines. Cells were 24hrs precultured in a concentration of a 1×10^4 cells/well in 96 tissue culture plates. Culture media were replaced by100 μl of the different tested materials. BV and nanocapsulated BV were added to pre-cultured plates then2 fold serially diluted. Plates were incubated for 24hr post treatment to permit trapped bee venom release. Treated media were removed and 50µl of MTT stain used as 0.5mg/ml added to each well and left for 4hr. Then 50µl DMSO was added to each well and absorbance was read at 570nm by LERX-800 ELISA reader (Biotech, USA).

Histopathological examination: The Ca-CO-2, MCF-7 and HEp-2 cell lines were treated with IC_{50} concentrations of BV, B-CS-NPs and BV Loaded CS-NPs, and then subjected to histological examinations to identify hisopathological changes appeared in treated cells. Negative control cells were considered (Mastropietro *et al*, 2015).

Slide preparation: Three cell lines were seeded in 24 wells plate (100µl cell suspension of each). The 24hrs post seeding, cells were treated with IC_{50} concentration of BV & BV loaded CS-NPs. Negative cell control was included. Plate was then incubated at 37°C for 24hr. post incubation; cells were trypsinized, resuspended in fresh medium and centrifuged at 2000 rpm for 10 minutes. 50µl of cell pellet was on glass slide; air dried for 10 minutes and fixed in methanol.

Hematoxylin and eosin: Fixed slides were dehydrated in descending grades of ethanol two changes of one minute each. Slides

were then immersed in filtered hematoxylin stain for 3 minutes and rinsed twice in distilled water. Slides immersed in filtered eosin stain for 5 seconds were washed with distilled water. Dried slides were immersed in xylene, mounted with Canada balsam and covered cover slips and left to dry.

Photomicrography and cytological evaluation: Fourteen microscopic fields of each slide were photomicrographed (oil immersion), by a digital video camera. Field selection was based on the presence of highest number of apoptotic cells. The photomicrographs were then evaluated for the presence of morphological criteria of apoptosis.

Results

Cytotoxicity assay; Bee venom inhibited growth of cancer cell lines in a concentration-dependent manner (viability% of cancer cells decreased with increased concentration). Magnitude of the inhibitory effect of bee venom alone was stronger against colon cancer (Caco2) and larynx cancer (HepG2) cells than breast cancer (MCF-7) cell lines (Fig. 2a), Susceptibility of cancer cell lines (Hep-2, MCF-7 & Caco2) towards nanoparticles after 48hrs (Fig. 2b). The viability% was concentration and cell line dependent. The IC_{50} values for BV & BV loaded nanoparticles on cancerous cell lines (CaCO-2, HEp-2 & MCF-7). IC_{50} values for BV were 9.2, 8.11 & 10.70µg/ml for CaCO-2, HEp-2 & MCF-7 cell lines, respectively. But, IC_{50} value of BV loaded nanoparticles recorded 7.7, 8.6 & 8.0 588µg/ ml for CaCO-2, HEp-2 & MCF-7 cell lines, respectively (Tab. 1). All cancer cells lines were sensitive to bee venom, empty and bee venom nanoparticles.

The morphology of different types of cancer cell lines was investigated under inverted microscope. In all types of cancer cell lines, control (untreated) cells were well adherent, homogeneously distributed in the culture field exhibiting a polygonal shape with distinct boundaries and homogenous cellular contents. On the other hand, morphological observations of treated cancer cells from all cell lines at the higher concentrations of tested materials (BV and BV loaded nanoparticles) showed destruction of cell sheet, cell shrinkage, rupturing and detaching from the surface of tissue culture (Figs, 3, 4& 5).

Histopathological examination: In all cell lines, control (untreated) cells they were regular cells with regular cellular and nuclear membranes and hyperchromatic nuclei (Figs. 6a; 7a & 8a). Cancer cells lines treated with IC_{50} of BV showed Shrunken apoptotic cell (black arrow), peripheral condensation of chromatin (Yellow arrow) and nuclear fragmentation (red arrow)(Fig. 6b), apoptotic bodies (black arrows) and necrotic cell debris (yellow arrows) (Fig. 7b) and shrunken apoptotic cells with irregular cellular and nuclear membranes (Yellow arrows) and peripheral condensation of chromatin (Red arrow), Swollen necrotic cell with swollen nucleus (black arrows) (Fig. 8b). Cells treated with IC_{50} of BV loaded NPs showed small shrunken apoptotic cells (yellow arrows) and peripheral chromatin condensation (black arrow) (Fig: 6c), apoptotic shrunken cells with shrunken nuclei (green arrow). Some cells were irregular (red arrows) and nuclear membranes (black arrows) and peripheral condensation of chromatin (orange arrow) (Fig. 7c) and apoptotic cells with peripheral chromatin condensation (yellow arrows) and nucleolar segregation (ted arrow) (Fig. 8c).

Table 1: IC_{50} of BV & BV loaded chitosan nanoparticles on different cancer cell lines (CaCO-2, HEp-2& MCF-7)

Tested material	Cell line	IC_{50} (µg / ml)
BV	*CaCO-2*	9.2
	HEp-2	8.11
	MCF-7	10.70
BV loaded NPs	*CaCO-2*	8.7
	HEp-2	7.3
	MCF-7	10

Discussion

Several parameters affect the cytotoxic profile of CS-NPs, such as their size, surface properties and degree of deacetylation (46%-88%) (Loh *et al*, 2010). Results of the present study showed that BV loaded chitosan nanoparticles have a good anti proliferative effects against the studied cell lines, where HEp-2 cells were more susceptible to BV loaded nanoparticles than MCF-7. These results agreed with Saha *et al*. (2014) who stated that cytotoxicity of gold nanoparticles (GNPs) conjugated with a protein toxin from the Indian cobra *Najakaouthia*venom (NKCT1) on leukemic cells (U937 and K562) was more obvious than native NKCT1. Also, N-succinyl chitosan nanoparticles conjugated with lipid showed better antitumor effect on K562 cells (Yang *et al*, 2009; Luo *et al*, 2010). In the same manner, cisplatin and cisplatin loaded chitosan nanoparticles exerted cytotoxic effect on HeLa cells in a dose dependent manner. The cytotoxic effect of cisplatin loaded nanoparticles is comparable to that of cisplatin alone (Uztüzün *et al*, 2015). Also, curcumin-loaded alginate nanoparticles (CS-ALG-NPs) displayed significant antitumor activity compared with the free curcumin (Ahmadi *et al*, 2017). In the same regard, synthesized *ethanolic extract of Gymnema sylvestre* leaves loaded chitosan nanoparticles (GSCNPs) and *Cinnamomum zeylanicum* bark extract loaded chitosan nanoparticles (CZCNP) exhibited dose-dependent cytotoxicity against human cervical cancer (SiHa) cell line, with inhibitory concentration (IC_{50}) values of 102.17 µg/ml, 87.75µg/ml, 132.74µg/ml and 90.35µg/ml for *Gymnema sylvestre* (GS) leaf extract, GSCNPs, CZBE and CZCNPs, respectively (Sujima *et al*, 2016). Moreover, it has been observed that CS-NPs of a molecular weight of 10-213 kDa at a size of 110-390 nm and degree of deacetylation; 46%-88% show comparable cytotoxicity profiles against A549 (human adenocarcinoma) cells with cell viability generally not affected by sample concentrations lower than 740 µgml-1 (Ma and Lim, 2003).

In contrast, this was different when using the MGC803 human gastric carcinoma cell line where the IC_{50}value was 16.2 and 5.3 µgml-1 after 24h & 48h, respectively, in incubation with CS with a size of 65 nm and high charges of 51mV (Qi *et al*, 2005). Treatment of liver cancer cell line (HepG2) with different concentrations of 150 nm diameter chitosan nanoparticles (CS-NPs) did not show alteration of cell morphology after 24 h of cell exposure. Also, when cells were treated with 100 µgml-1 of CS-NPs, 12% of them were killed and IC_{50} reached 239 µgml-1 after 48h of cell exposure (Lotfy *et al*, 2016). In this regard, cytotoxicity of 40 µM ferulic acid-loaded chitosan nanoparticles (FA/CS-TPP NPs) against ME-180 cervical cancer cell lines was found to be higher as compared to 40 µM native FA (Panwar *et al*, 2016).This indicates that small size, high positive charges and the type of biologically interacting cells and cellular uptake dramatically affect the cytotoxic effect of CS-NPs.

There are two main strategies by which chemotherapeutic agents are capable of exerting their effect: (1) induction of apoptosis and (2) cell cycle inhibition. Many of the compounds act at multiple steps in the cell cycle, and their effects may be cytostatic or cytotoxic, depending on the cell cycle status of target cells (Shapiro and Harper, 1999).

Apoptosis plays an important role in cell cycle. In this context, the present study indicated that at IC_{50} concentrations of the BV and BV loaded nanoparticles induced apoptosis in treated cancerous cells under study. The morphological changes of accompanied with apoptosis ranged from the membrane blebbing, cell shrinkage, nuclear chromatin condensation, shape irregularity, apoptotic bodies in late stage, nucleolar fragmentation, shrunken nucleus and some necrotic features, while control cells showed normal pathway of mitotic divisions in the form of obvious cell membranes and nucleus, when stained using Hematoxylin and Eosin (H&E)

and visualized using light microscope. Literature reports that chitosan nanoparticles owing to their positive surface charges adsorb with a high affinity onto negatively charged tumor cell membrane (Qi *et al*, 2005).

In the present investigation, BV loaded CSNPs would have bind to the tumor cell membrane via electron interactions, entered the cell through endocytosis and released the encapsulated BV into cytosol (endosomal burst). Due to increased bioavailability, BVmay exhibit its antitumor effect through up regulation of P_{53} and Bax and down regulation of Bcl2 genes which would eventually lead to cell death. Chitosan nanoparticle-conjugated bee venom preferentially delivered into the nucleus inside the cells for eliciting a better therapeutic effect than native bee venom, where chitosan can escape the endosomes offering high potential for nuclear delivery. Molecular entry into the nucleus occurs through the nuclear pore complexes; the efficiency of which is dependent on NP size and the presence of nuclear localization sequence (NLS) (Tammam *et al*, 2015). The above-mentioned results agreed with that of another study who found that chitosan enveloped ferulic acid (FA) nanoparticles induced apoptotic morphological changes such as cytoplasmic remnants and damaged wrinkled cells in human cervical cancer cell line (ME-180)when visualized using scanning electron microscopic and fluorescent microscopic techniques (Panwar *et al*, 2016). Besides, cisplatin and cisplatin loaded chitosan nanoparticles exert apoptotic and necrotic effects on HeLa cells in a dose dependent manner. Apoptotic indexes generated from caspase-3 staining and double staining was compatible and found as 60±5%, 53±4% for cisplatin and cisplatin loaded nanoparticles, respectively, while the necrotic index was 64.2±3%, 60.3±2% for cisplatin and cisplatin loaded nanoparticles (Uztüzün *et al*, *2015*). Ferulic acid (FA) from Partheniumhystero-phorus and FA encapsulated nanofibers were found to exhibit anticancer potential against different cancer cell lines (Panwar *et al*, 2016). Also, curcumin-loaded NPs significantly suppressed proliferation and promoted the induction of apoptosis in human cervical epithelioid carcinoma cancer cells (Ahmadi *et al*, 2017). Cell apoptosis study revealed that Paclitaxel chitosan nanoparticles (PTX-CS-NP) treatment resulted into enhanced (almost double) late cell apoptosis than native paclitaxel. Hence the developed nanoparticulate formulation resulted into improved anticancer efficacy of paclitaxel (Gupta *et al*, 2017). Similar results were obtained in case of CNPs that used as Tamoxifen carriers against breast cancer cells (Vivek *et al*, 2013). Also, it was reported that an enhanced rate of apoptosis by PLGA 50-50 nanoparticles against Glioma (U87MG) and breast adenocarcinoma (MCF-7) cell lines occurred (Nair *et al*, 2011).

Conclusion

BV loaded NPs exhibited a good cytotoxic activity. The surface charge of chitosan was the major factor in the cytotoxic activity due to the electrostatic ionic interaction between the negatively charged groups of tumor cells and the positively charged amino groups of chitosan in addition to cytotoxic effect of bee venom itself, which strongly suggest that eco-friendly CNP-based drugs could be developed appropriately for the treatment of cancer cells without any effects. BV loaded NPs offer a promising new approach to drug delivery in the treatment of cancer. Nano-capsulated bee venom showed anticancer potential than BV itself. Also, Nano capsulation could enhance the anticancer potential that was cell type and concentration dependent manner. Further study should focus on possible *in vivo* use of BV loaded NPs.

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Explanation of figures

Fig. 1: Schematic representation of chitin and chitosan extraction process

Fig. 2: Relationship between concentrations and residual cell viability percentage of different cancer cell lines post treatment with: (a) BV and (b) BV loaded nanoparticles (BV loaded NPs)

Fig. 3: Morphological characteristics of unstained treated and untreated CaCO-2 cells (A): untreated cells, (B): Toxic effect of bee venom. (C): Toxic effect of Empty nanoparticles. (D): Toxic effect of BV loaded nanoparticles.

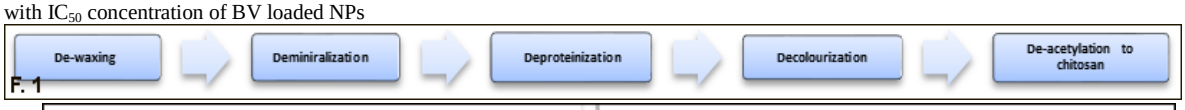
Fig. 4: Morphological characteristics of unstained treated and untreated HEp-2 cells(A): untreated cells, (B): Toxic effect of bee venom. (C): Toxic effect of Emptynanoparticles. (D): Toxic effect of BV loaded nanoparticles.

Fig. 5: Morphological characteristics of unstained treated and untreated MCF-7 cells (A): untreated cells, (B): Toxic effect of bee venom. (C): Toxic effect of Empty nanoparticles. (D): Toxic effect of BV loaded nanoparticles.

Fig. 6: Histopathological observations of CaCO-2 cells: (a) control cells; (b) cells treated with IC_{50} concentration of BV; and (c) cells treated with IC_{50} concentration of BV loaded NPs

Fig 7: Histopathological observations of HEp-2 cells: (a) control cells; (b) cells treated with IC_{50} concentration of BV; and (c) cells treated with IC_{50} concentration of BV loaded NPs

Fig 8: Histopathological observations of MCF-7cells: (a) control cells; (b) cells treated with IC_{50} concentration of BV; and (c) cells treated with IC_{50} concentration of BV loaded NPs



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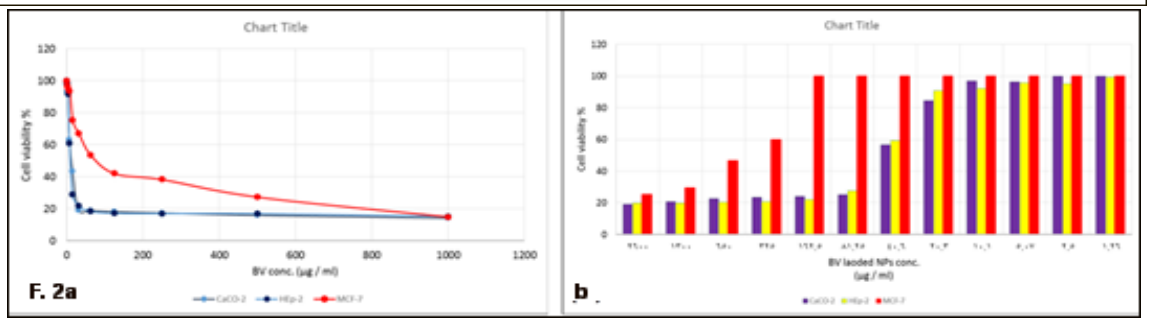

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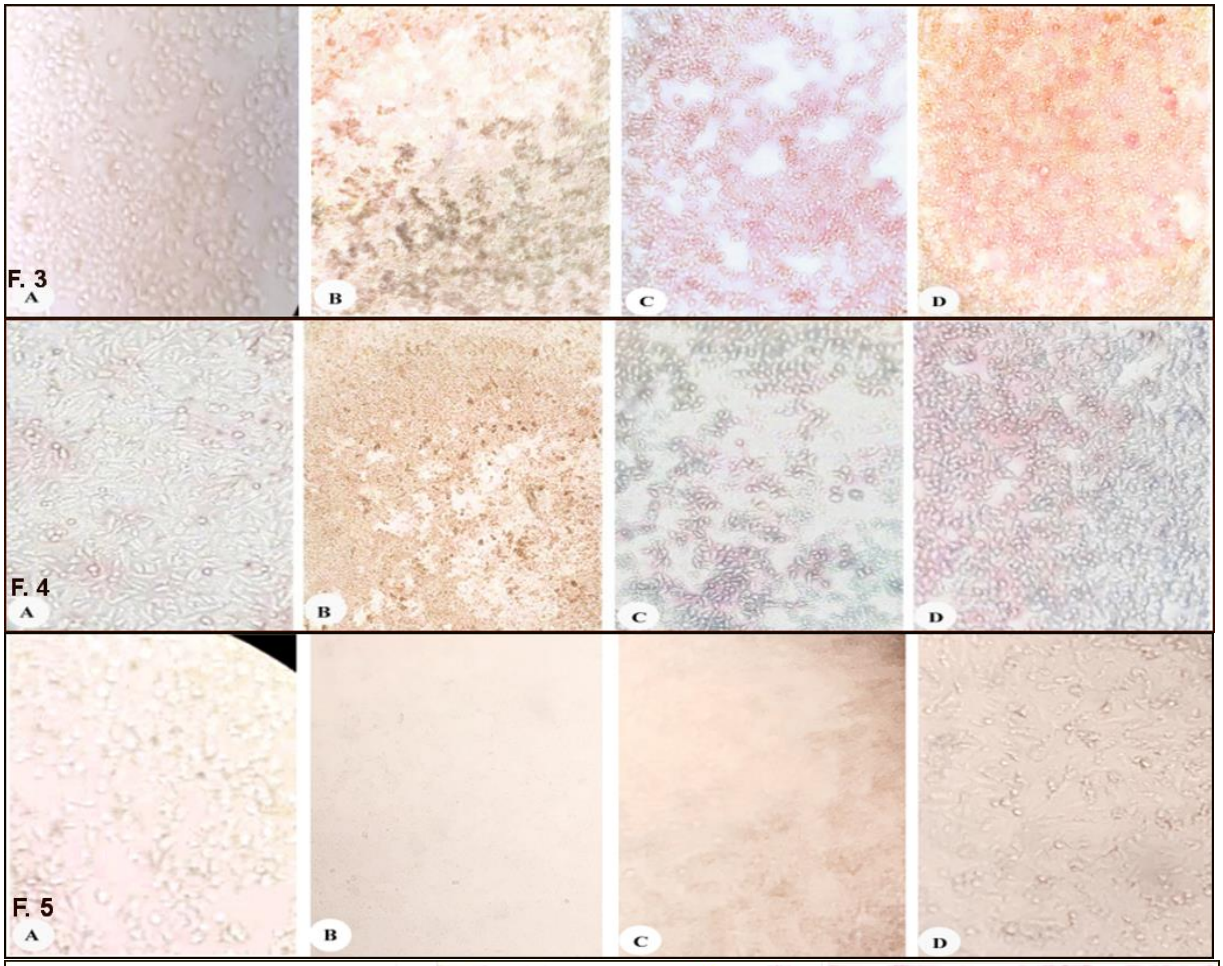
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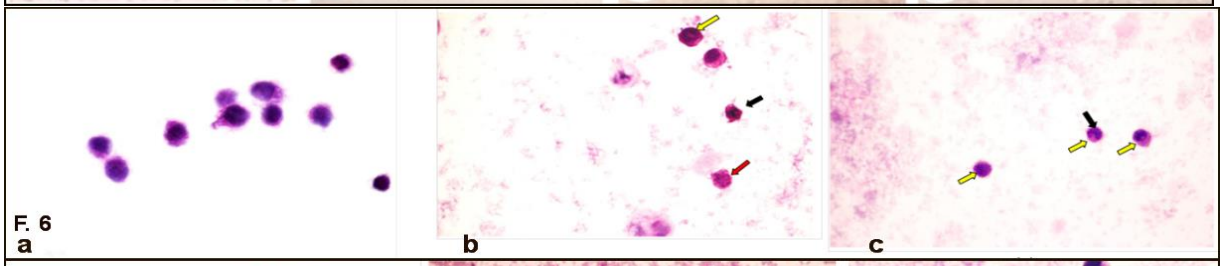
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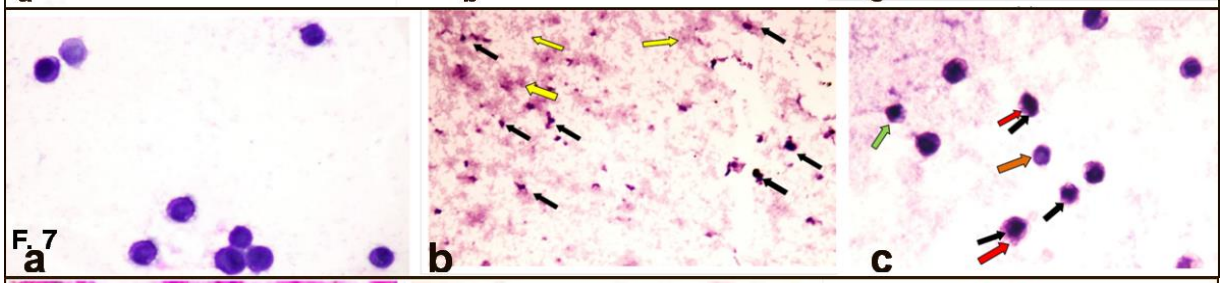
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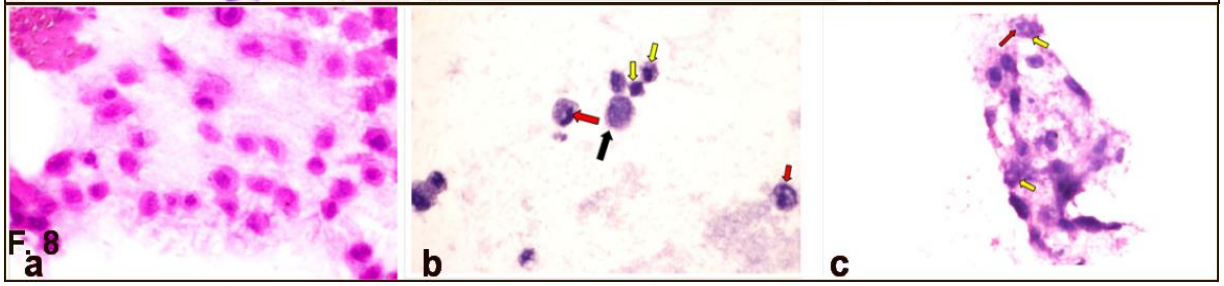
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