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DETERMINATION THE EFFECT OF *HELICOBACTER PYLORI* TOXIN ON NORMAL AND CANCER CELLS LINES

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ABSTRACT

The samples were collected from 120 patients who underwent endoscopy (53 males and 67 females) aged 11-76 years. Gastric biopsies were taken from all patients for detection of *H. pylori* by rapid urease test, culture, and PCR. The *H. pylori* toxin was extracted and tested on experimental animals (Balb/c) mice and two types of cell lines Normal Breast and Esophagus Cancer. The toxin was analyzed through (GC-MS) to determine the components effect. The results indicated that *H. pylori* toxin have the ability to inhibit DNA synthesis in humans Esophageal Cancer cell lines and stopping the cell's proliferation in the concentration (1000 µg/ml), while there was no effect of the toxin on Normal Breast cells and there was an increase in the vitality rate concentration (1000 µg/ml).

I. INTRODUCTION

H. pylori is a gram-negative microaerophilic bacteria found in the mucosal layer of the human stomach. It is a human pathogen that causes chronic inflammation, gastritis, gastric atrophy, and gastric adenocarcinoma by secreting urease to counteract the stomach's harsh environment [1].

Marshall and Warren were the first to characterize spiral-shaped microaerophilic bacteria (1983), which causes many diseases has opened up new paths for research and altered perceptions of *H. pylori*-mediated disorders diagnosis and treatment [2].

The majority of infections are thought to be transmitted by fecal-oral, oral-oral, or gastro-oral routes. Variable outcomes of *H. pylori* infection are determined by factors such as strain-specific bacterial components, inflammatory responses triggered by host genetic diversity, and environmental factors, all of which have an impact on pathogen-host interactions. It has also been postulated that *H. pylori* may play a role in the oral cavity [3].

Invasive and non-invasive diagnostic procedures for *H. pylori* isolation and identification exist. Invasive procedures need endoscopy to acquire biopsies of stomach tissues or mucus samples. Non-invasive procedures include urea breath testing, serology, stool antigen testing, whereas invasive procedures include histology, culture, urease test, and molecular methods [4].

This bacterium possesses multiple virulent mechanics. *CagA* and specific alleles of *VacA* are known as the two best-studied virulence factors linked to the occurrence of stomach cancer [5].

Due to the importance of these bacteria and the possibility of using the toxin secreted by them in dealing with the most important and the most dangerous types of cancer, which studied on cell lines are ubiquitous tools for experimental biomedical research, based on that this study designed to evaluate the role of *H. pylori* in gastrointestinal tract infection in different samples and different methods by the following objectives:

- Isolating and identifying *H.pylori* from biopsy in Basrah governorate using culturing and PCR (16SrRNA)
- Determining the effect of the toxin isolates by histological study experimentally using injection mice assays.
- Clarified the effect of *H. pylori* toxins on cell lines normal and cancer.

II. MATERIAL AND METHODS

- **Samples collection:** (120) patient's samples were analyzed, 53 male and 67 female who suffering from abdominal problems, clinically diagnosing symptoms of peptic ulcer disease such as upper abdominal pain, acidity, nausea, and vomiting through the endoscopic process in AL- Sadder Teaching Hospital in Basrah, GIT unit from November 2020 to May 2021. From each patient, two stomach biopsy samples collected from antrum.

- **Isolation of *H. pylori:*** Biopsy samples were homogenized by using sterile fine glass rod, then streaked on chocolate agar medium by using a sterile cotton swab, and incubated in microaerophilic conditions in the anaerobic jar for 5-7 days at 37 °C [6].

- **Biochemical tests**: Probable pure colonies were chosen based on Gram staining and culture features and evaluated for catalase, oxidase, and urease production [7].

- **Genotypic Characterizations of the Isolates:** Genomic DNA was extracted and purified from bacterial according to the protocol of presto ™ Mini gDNA bacteria kit

- **Visualization of total DNA on agarose gel:** DNA was visualized by agarose gel electrophoresis [8].

- **Polymerase Chain Reaction (PCR)**

16SrRNA PCR Isolate Detection: Twenty isolates were identified biochemically from biopsy samples and were chosen at random to be validated using primers specifically designed for the identification of *H. pylori* based on the 16SrRNA sequence [9]. The forward primer sequence for the 138 bp product of the 16SrRNA sequence is 5 GCGACCTGCTGGAACATTAC 3 and the reverse primer sequence is 5 CGTTAGCTGCATTACTGGAGA 3

- **Extraction of the toxin and identification it by (GC–Mass):** For extraction of *H. pylori* toxin, the strains were inoculated into 250 ml Brain heart infusion broth and incubated at 37°C in a rotatory incubator-shaker at 150 rpm 48 hr. The cultures were then put in cool centrifuged 40°C at 6000 rpm for 30 min, and the respective supernatants were carefully separated and poured into new vials and stored at room temperature [10].

- **Mice injected assay**

- Three groups of 12 weeks old (males and females) mice six in each group namely A, B, and C were injected with *H. pylori* toxin. In group A: six mice were injected intraperitoneal (I.P.) with a concentration of 155 mg/ml using a syringe. In group B: six mice were administered orally with a concentration of 155 mg/ml of *H. pylori* crude toxin using a separate micropipette and all groups were kept in incubation at 37 °C for a week to observe toxic effects. The mice of group C were kept as a control in the same environment [11].

- **Histological study** was handled by the University of Basra's Science College. Through routine histopathology examination work. Following fixation, the tissue block is embedded in paraffin slices [12].

- **Cell line viability assay**: Work has been done in Laboratories Iraqi biotech Company for Scientific Research, cell culture unit, using two types of cell lines obtained from the Iraqi Center of Cancer and Medical Genetics Research(ICCMGR), Al-Mustansiryia University, and maintained in RPMI-1640 supplemented with 10% Fetal bovine, 100 units/mL penicillin, and 100 µg/mL streptomycin. Cells were passaged using Trypsin-EDTA reseeded at 70% confluence twice to third a week and incubated at 37 °C and 5% CO_2 [13].

- **Cytotoxicity Assays:** To determine the cytotoxic effect, the MTT cell viability assay was conducted on 96-well plates. Cell lines were seeded at 1 × 104 cells/well. After 24h or a confluent monolayer was achieved, cells were treated with the difference toxin concentrations (500& 1000) µg/ml. Cell viability was measured after 72 hrs. of treatment by removing the medium, adding 28 µL of 2 mg/mL solution of MTT (and incubating the cells for 2 h at 37 °C. After removing the MTT solution, the crystals remaining in the wells were solubilized by the addition of 100 µL of DMSO (Dimethyl Sulphoxide) followed by 37 °C incubation for 15 min with shaking [14]. The absorbency was determined on a microplate reader at 620 nm (test wavelength); the assay

was performed in triplicate. The inhibition rate of cell growth (the percentage of cytotoxicity) was calculated as the following equation:

Proliferation rate as (PR)= B/A*100 where A is the mean optical density of untreated wells and B is the optical density of treated wells and IR= 100- PR [15].

- **Statistics analysis:** The results were evaluated by using the chi-square test Statistical Package for Social Science (SPSS) to determine the statistical significance of the data, and significance was assumed at $p< 0.05$.

III. RESULTS AND DISCUSSION

From all patients under study table (3.1), 48 out of 120 (40%) infected with *H. pylori* and (60%) recorded as non-*H. pylori* infected. And according to gender, 26(54.2 %) out of 53 (44.2%) were males, while 22 (45.8 %) out of 67 (55.8%) were females. The differences were not statistically significant (P> 0.05).

Table (3.1): patient *H. pylori* and Non – *H. pylori* according to Gender

Gender	Patient		*H. pylori*		Non –*H. pylori*		P-value
	NO.	%	NO.	%	NO.	%	
Male	53	44.2	26	54.2	27	37.5	
Female	67	55.8	22	45.8	45	62.5	0.092
Total	120	100.0	48	100.0	72	100.0	

The highest incidence of *H. pylori* infection was 10(55.6%) among patients over 60 years old, while the lowest incidence of infection was 2(40.0%) among patients aged 20 years and under. The findings demonstrated that *H. pylori* infection increased with age, but the difference was not statistically significant (P >0.05).as shown in table (3.2).

Table (3.2): Distribution of patients *H. pylori* and non-*H. pylori* according to age groups.

Age groups	**Patients**		***H. pylori***		***Non-H. pylori***		**P-value**
	NO.	**%**	**NO.**	**%**	**NO.**	**%**	
Years 20<	5	4.1	2	40.0	3	60.0	
20-29 Years	23	19.2	6	26.1	17	73.9	
30-39 Years	30	25.0	12	40.0	18	60.0	
40-49 Years	24	20.0	8	33.3	16	66.7	0.422
50-59 Years	20	16.7	10	50.0	10	50.0	
>60 Years	18	15.0	10	55.6	8	44.4	
Total	120	100.0	48	40.0	72	60.0	

These findings were similar to Pounder [16] and Ugwuga [17], who claimed that *H. pylori* infections have been demonstrated worldwide and affect all age groups. It is estimated that half of the world's population is infected; similar findings were found by Amer *et al*. [18], who studied *H. pylori* infections in immunocompromised patients and discovered that there was no significant difference in sex between the two groups. In addition, Wang *et al*.[19] showed that the prevalence of *H. pylori* was (37.2%) among women and (34.9%) among males.

To quickly detect *H. pylori* infection Rapid Urease Test (RUT) was used. A pink tint result indicates the presence of *H. pylori* figure (3.1). Rapid urease tests have been widely used because they are simple, cheap, easy to carry out and provides quick results.

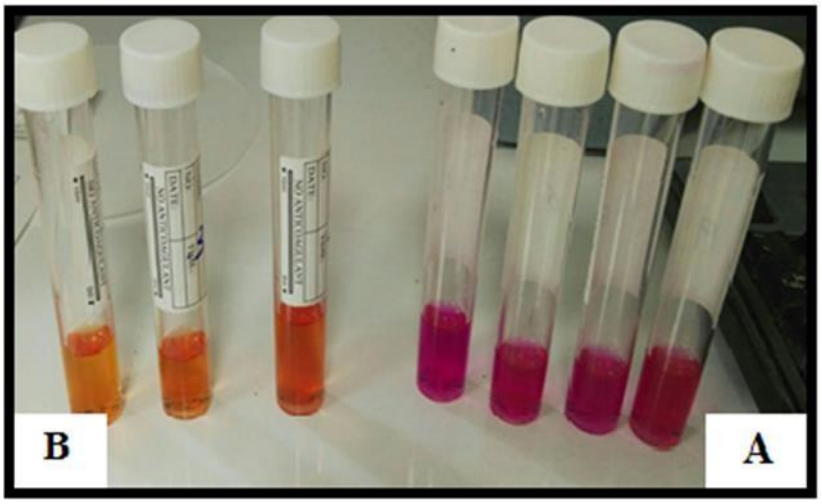


Figure (3.1) Rapid urease test (RUT)

A- Positive RUT

B- Negative RUT

The current findings revealed that 20(41.6 %) of *H. pylori* was isolated by using the culture method. In Gram stain, the bacterium appeared Gram-negative spiral form or curved rods as seen in figure (3.2). In fact, isolating *H. pylori* from biopsy specimens was a difficult process due to the fastidious nature of bacterium and may be conflicted several factors which are difficult to control, causes difficulty with the culturing of the organism, such as patch distribution of organism on the gastric mucosa, contamination of biopsy forceps, presence of oropharyngeal flora, loss of viability of organism during transportation as well as intake of the proton pump inhibitors (PPI) which have an indirect antibacterial effect. All these may be responsible for a negative predictive value associated with a culture of *H. pylori*. This discovery was comparable to Ieni *et al*. [21].

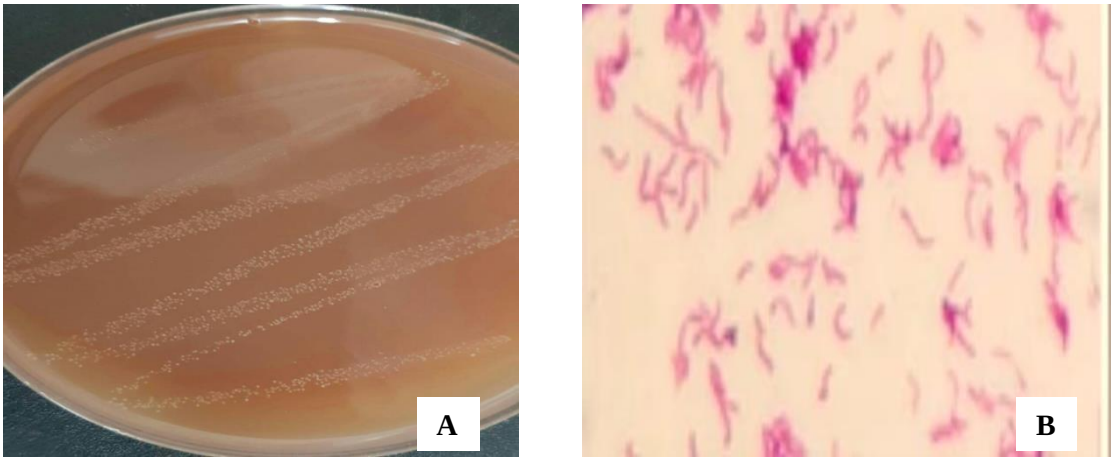


Figure (3.2): Colonies of *H. pylori* isolated from biopsy specimen

A- *H. pylori* on chocolate agar medium
B- Gram stain of *H.pylori*

According to culture technique, morphology, and biochemistry, only 20 isolates from 48 patients exhibit positive PCR results for the 138 bp region of the 16SrRNA gene. PCR findings for 16SrRNA based on primers generated bands on an agarose gel corresponding to a 138 bp product, identifying isolates as *H. pylori* when compared to the molecular ladder.

PCR was also employed to identify *H. pylori* directly on gastric biopsy samples from 48 patients, with amplification of the 16SrRNA gene targeting the 138 bp domain performed on extracted DNA from gastric biopsy specimens. The PCR results were run on a 1.5 % agarose gel electrophoresis, which revealed bands of amplified 16SrRNA DNA. In 48 biopsy specimens, the predicted 138-bp amplification was identified as shown in figure (3.3).

H. pylori has been diagnosed in clinical samples using genetic tests based on PCR, which allows for the precise detection of nucleic acid. As a result of its extreme sensitivity. When an organism is present in small numbers, grows slowly, or is difficult to detect, PCR is an appropriate method for diagnosis. Simultaneously, the process is highly vulnerable to inhibitors induced by contaminants found in clinical samples and hence may yield erroneous results [22]. The PCR technique is a more advanced and precise method for detecting *H. pylori* infections based on the bacteria's DNA [23]. As a result, genetic testing was used to determine the final *H. pylori* diagnosis test, amplification of 16S RNA, and alignment of DNA sequencing with gene bank on NCBI.

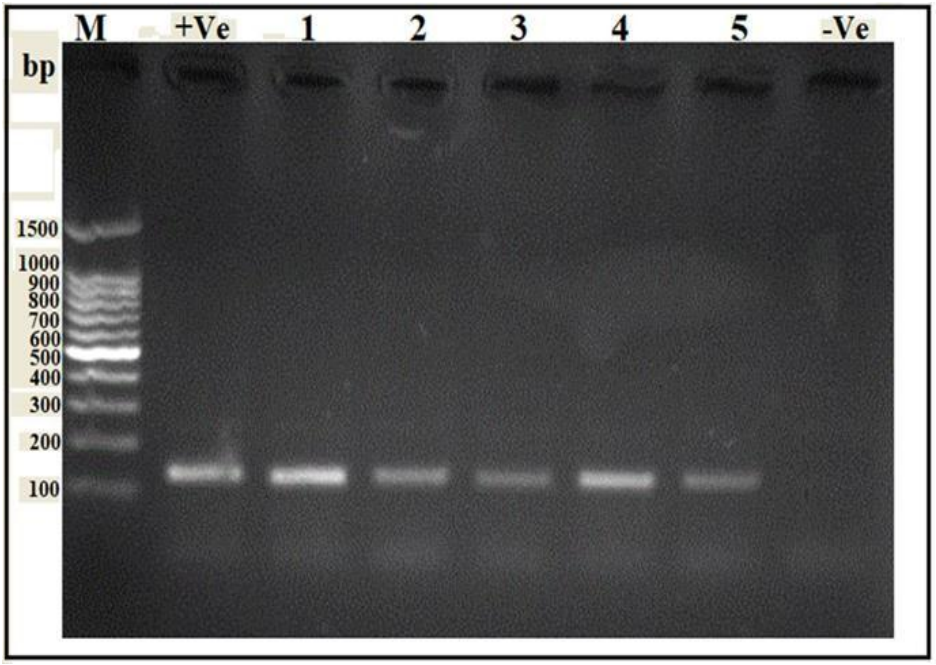
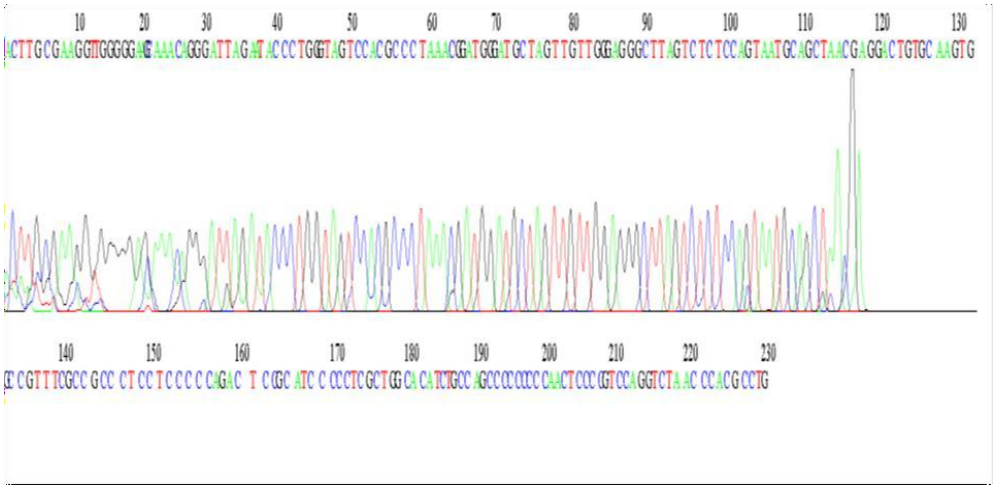


Figure (3.3) amplification of 16s rRNA gene of *H.pylori* clinical isolates. M: Molecular size marker 100 bp. +Ve Positive control and –Ve Negative control. Lanes 1, 2, 3, 4, 5, represented of 16S rRNA gene at 138bp band from DNA of *H.pylori* (1.5 % agarose gel at 60 volt for 1 hour)

The PCR - products of the 16S rRNA gene was sequenced using the same primer as described for the amplification process. Sequences were aligned in the Gene Bank database using the BLASTN. program at the National Center for Biotechnology Information (NCBI). The results showed that the isolates which have been detected by sequence were identified in the gene bank in (96%) figure (3.4). The species recorded in the current study close to *Helicobacter pylori* strain 169-C-EK8 chromosome, complete genome. 1611628 bp DNA circular BCT 13- JUN-2019.



Through using (GC- MS) device to diagnosis the effective compounds of *H. pylori* crude toxin extraction the results showed that the chemicals compounds appeared in the analysis are phenolic acids and flavonoids which are the most common phenolics with antioxidant action [24].

In the present study, the histological effect of *H. pylori* toxin by using mice injection assays showed that from six infant mice two mice that have been injected (I.P.) with 155 mg/ml toxin died after 48 hours of injection. This result confirms that *H. pylori* isolates produce a toxin. While all mice which have been administered toxin orally still survived. On the other hand, all the control mice have remained to survive during the experiment. The obvious disparities between control mice who were not injected with toxin and infected mice are seen in figure (3.5).

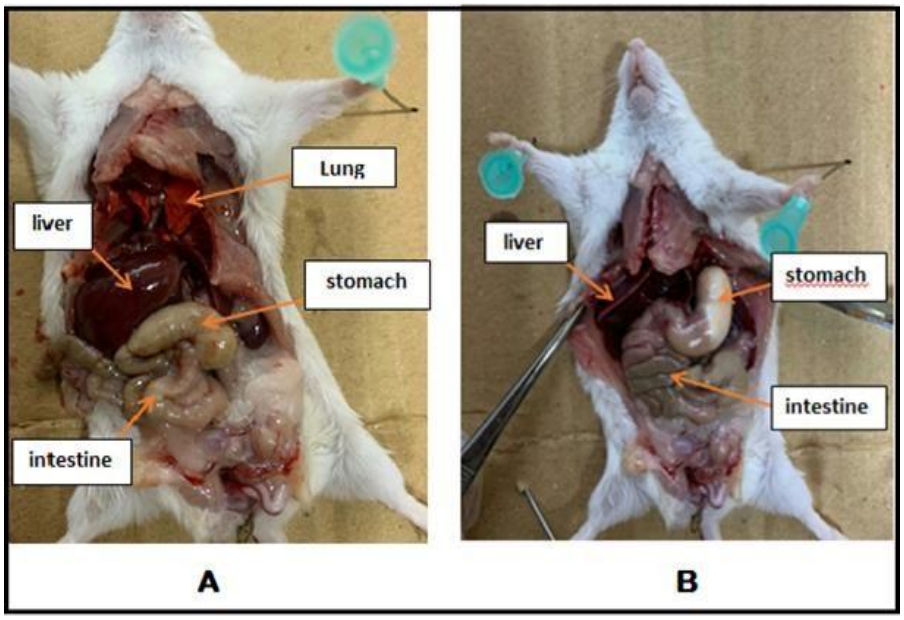


Figure (3.5) In a comparison image of a normal mouse "control" and a mouse treated with *H. pylori* crude toxin, the toxin-induced macroscopically changes in organs such as enlarged, hypertrophied liver and spleen, as well as deteriorated characteristics in the stomach, intestine lung and kidney when compared to a normal mouse , A- Injected toxin, B- Control.

The cytological microscopic examination results of stomach showed dilation of the lumen of gastric gland and metaplasia of lining gastric gland presence of foci of inflammation and the infiltration of large numbers of inflammatory cells compared with stomach mouse control section which showed normal stomach wall composed of an epithelial layer, muscular mucosa layer, and a submucosa layer as shown in figure (3.6, 3.7).

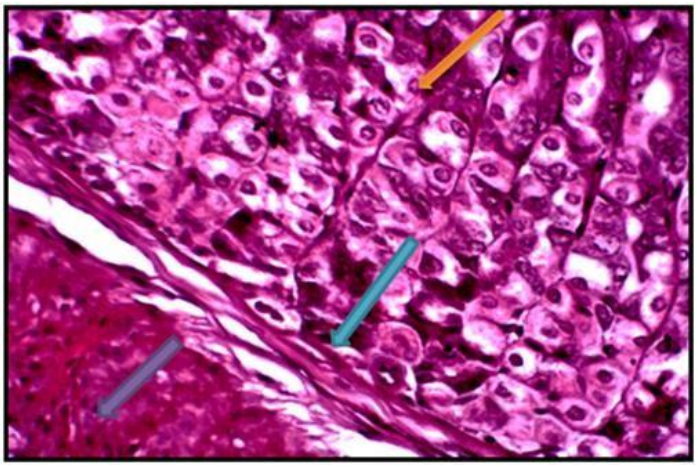


Figure 3.6: Section of control stomach shows normal epithelial layer ( ) muscular mucosa layer( ) submucosa ( ) (H& E stain ,100X)

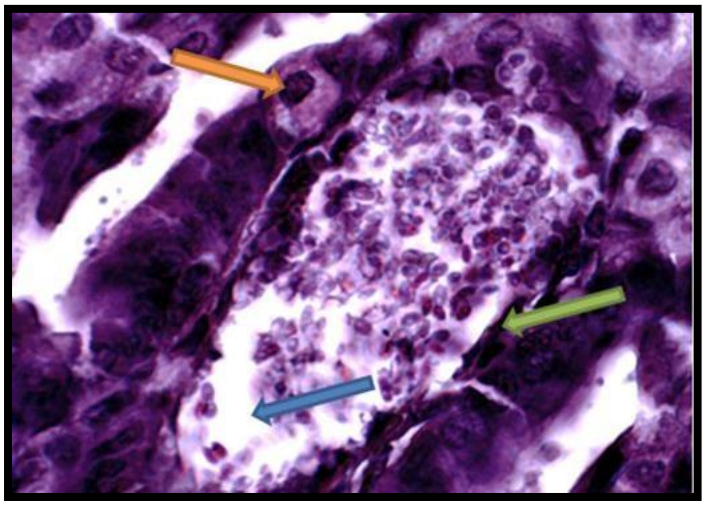
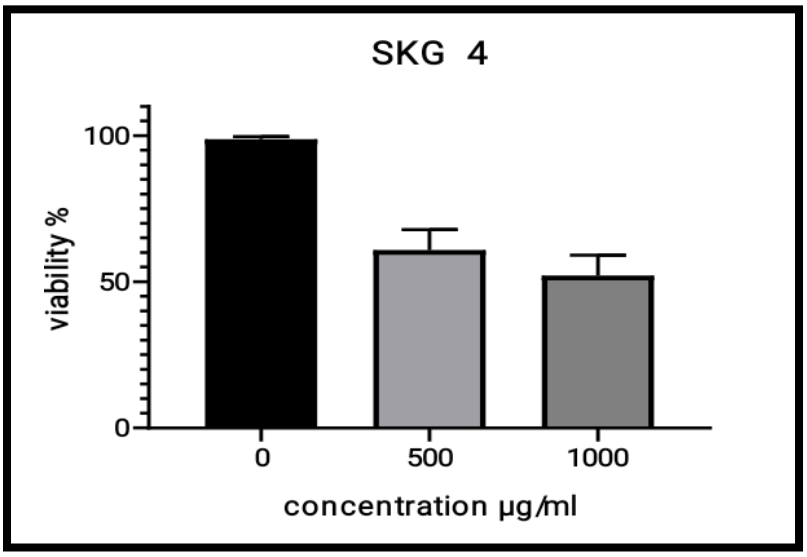


Figure 3.7: Section of injected orally stomach (155mg/ml) shows dilation of lumen of gastric gland ( )and metaplasia of lining gastric gland ( ) parietal cell ( ) (H& E stain ,100X)

The normal stomach consists of three layers of muscle: an outer longitudinal layer, a middle circular layer, and an inner oblique layer. The inner lining consists of four layers: the serosa, the muscularis, the submucosa, and the mucosa. The mucosa is densely packed with gastric glands, which contain cells that produce digestive enzymes, hydrochloric acid, and mucus [25].

Two types of cells lines were used SKG and HBL100 to investigate the effect of *H. pylori* toxin extract on the viability of the cells. 5 out of 20 isolates choosing randomly, with two concentrations (500& 1000) µg /ml from each toxin used to study the viability of cells lines.

The results in SKG cell line showed the viability of treated cells in comparison with control cells dependent on concentrations as shown in Figure (3.8). H. pylori toxin could inhibit esophagus cancer cells growth by inhibiting DNA synthesis in human esophagus cancer cells and regulating cell proliferation. The toxin prevents the hyperproliferative and neoplastic development of Esophagus cells associated with the initiation and progression of cancer This study is approximately similar to study the effect of ST Enterotoxin Escherichia coli on colon cancer cell line [10].



Figure(3.8) viability ratios of SKG cell line treated with *H. pylori* toxin

According to cytomorphological death, the microscopic examination of the SKG cell line stained with AO/EB dye showed that the untreated cells were stained with the green color of AO which indicates intact cells, while the cell treated with H. pylori toxin stained with the yellow or red color of EB dye which indicates cell death. AO/EB assay use for observing the differential uptake of fluorescent DNA-binding stain AO/EB. cells staining with red indicates that the necrotic and apoptotic cells suffered from a defect of permeability, which causes to penetration of EB through the plasma membrane and nuclear envelope then connect with the nuclear material of the cell. The yellow cells are ongoing to death as pro-apoptotic or pro-necrotic cells. Untreated cells were stained with the green color of AO stain, an indication of the integrity of their membranes as shown in figure (3.9).

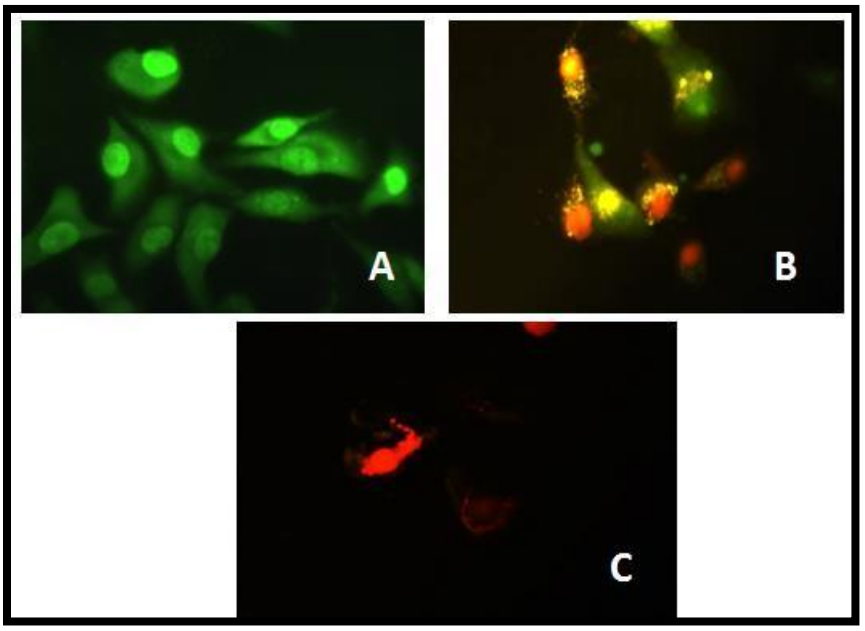


Figure (3.9) :AO/EB staining cells - A- Untreated SKG cells; B- cells treated and affected with toxin *H . pylori*; C : dead cell

The DNA fragmentation induced by *H. pylori* toxin showed that a perfect DNA ladder style was clear in a time and concentration-dependent manner. This may indicate that the cells were apoptosis or necrosis occurred as shown in figure(3.10), shows genomic DNA isolated from the SKG cell line.

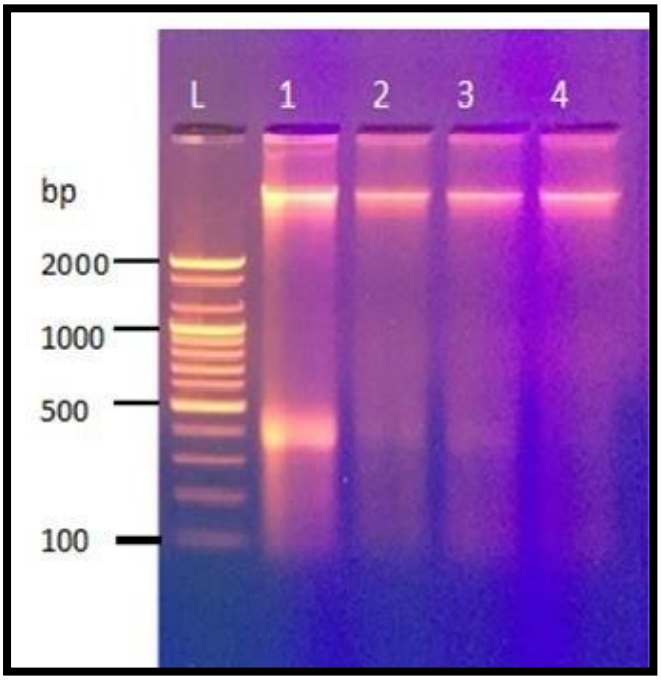
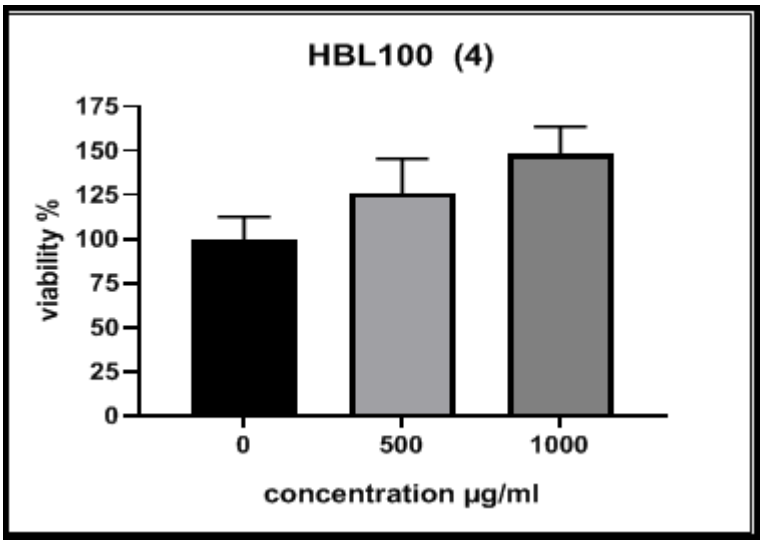


Figure (3.10) Effect of treating SKG cells with *H. pylori* toxin on DNA. L represents the DNA ladder (Bioneer) and 1 represents untreated cells whereas 2-4 represents treated cells with toxin *H.pylori*. After exposure to SKG cells at 1000 µg/ml concentration of extract, genomic DNA was extracted from untreated and treated cells using the cell culture protocol attached with a Geneaid kit according to the manufacturer's instructions. DNA was separated on a 1.5 % (w/v) agarose gel.

HBL-100 of the *H. pylori* toxin test there was no effect of the toxin on Normal Breast cells and there was an increase in the vitality rate concentration(1000 µg/ml), notes the continued division and proliferation of cells as shown in figure (3,11).



Figure(3.11) viability ratios of HBL-100 cell line treated with *H. pylori* toxin

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