Influence of Extremely Low frequency Magnetic Field on Proteus mirabilis bacteria

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ABSTRACT

Urinary tract infections (UTI) due to *Proteus mirabilis* strains represented nearly 90% of Urinary tract infection cases. In this study the effect of applying extremely low frequency square magnetic pulses ELF-SMP on *P. mirabilis* was investigated. While, it interferes with the bioelectric signals generated during the cell division process. Also, the effect of exposure on bacterial antibiotic susceptibility and morphological cellular structure was studied. The results indicated that there was a significant inhibition effect for *P. mirabilis* exposed to 0.6 Hz SMP for one hour and significant increase in antibiotic susceptibility to DNA, proteins, *β-lactamase* enzyme and cell-wall synthesis inhibitors. Additionally, results of dielectric relaxation and TEM indicated molecular and morphological changes. According to these aforementioned results, it was concluded that treatment of *P. mirabilis* cells with SMP at mentioned frequency acts on its cellular activity and structure of the bacteria which can make this method is a promising one in further in vivo applications.

***Key words**: UTI, Proteus mirabilis, ELF-SMP, antibiotic susceptibility*

1.INTRODUCTION

The urinary tract consists of kidneys, ureters, bladder, and urethra. The urinary tract infection is the infection or colonization of the urinary tract by pathogenic microorganisms. The most common uropathogens bacteria infect the urinary tract are: *Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus saprophyticus, Enterococcus faecalis* and *Enterobacter cloacae* **(Foxman, 2014)**.

P. mirabilis is the most common gram-negative pathogenic bacteria involved in a variety of community and nosocomial infections in humans, including acute urinary tract infections (UTI) particularly in patients with indwelling urinary catheters or in people with structural abnormalities of the urinary tract, (urolithiasis). Moreover, *P. mirabilis*

can also cause renal damage and infection in the respiratory tract, eye, ear, nose, skin, throat, burns, wounds and blood stream infections (BSI) **(Warren et al., 1982)**.

P. mirabilis is a rod-shaped bacterium that can be found in either of two cell types, a vegetative cell which is a short rod cell and a swarmer cell which is a highly elongated and hyper-flagellated cell. This differentiation is aroused by growth on solid surfaces and multiple inputs are sensed by the cell to initiate the differentiation process **(Morgenstein et al., 2010)**. It usually exists in the large intestine of many people. It's one of the useful microbes that forms a normal part of the gut flora. However, under certain conditions, the bacteria can escape from the intestine and cause a urinary tract infection. *Proteus* infections are usually associated with colonization and the formation of stones. The bacteria form solid crystals in the urine where, the crystals are joining together to make the stones. Small stones may escape the body on their own. Large stones may become restricted in the urinary tract and obstruct urine flow **(Coker et al., 2000)**.

P. mirabilis was susceptible to cephalosporins and β-lactam/ β-lactamase inhibitors, but it was resistant to nitrofurantoin and tetracycline . However, strains resistant to β-lactam mediated by acquiring β-lactamases emerged in 1990s **(Chanal et al., 2000)**. Among these β-lactamases, plasmid-borne extended-spectrum β-lactamases (ESBL) and AmpC β-lactamases were the most worrying enzymes because they result in resistance to nearly all penicillins, cephalosporins, β-lactams and quinolones and can extend to different species of Enterobacteriaceae **(Bush and Jacoby 2010)**. Nowadays the global tendency is to find out alternative treatment solutions instead of using antibiotics.

In a previous trial of *P. mirabilis* treatment, it was found that, increasing the intensity of the magnetic field shows a decrease of the microbial population of *P. mirabilis*. Also, it declared that the low-frequency magnetic field caused the decrease of colony forming units (CFU) in all exposed samples at a certain level of exposure **(Olajide and Nathaniel 2014)**. Numerous studies have been performed to investigate the remarkable effects of extremely low frequency (ELF) electromagnetic fields (EMFs) on cell functions **(Liang et al., 2006; Choi et al., 2008; Fox et al., 2008; Ruiz-Gomez et al., 2010; Inhan-Garip et al., 2011)**. Different responses have been observed on the cells **(Akan et al., 2010; Oncul et al., 2016)**, including proliferation and differentiation **(Grassi et al., 2004; Lisi et al., 2006; Vianale et al., 2008; Foletti et al., 2009)**, gene expression **(Goodman and blank 1998; Mattei et al., 2005; Goodman et al., 2009)**, alteration of functions of membrane receptors **(Bersani et al., 1997; Ke et al., 2008; Mattei et al., 2009)**, modulation in ion homeostasis **(Grassi et al., 2004; Piacentini et al., 2008)**, and free radicals generation **(Wolf et al., 2005; Zwirska-Korczala et al., 2005; Di-Loreto et al., 2009; Morabito et al., 2010)**. Particularly, it has been confirmed that exposure of the bacteria to ELF-EMF can alter its viability and growth rate **(Giladi et al., 2008)**, antibiotic susceptibility **(Segatore et al., 2012)**, and ultrastructural shape **(Inhan-Garip et al., 2011)** with frequency and amplitude dependency.

The aim of the present work was to study the effect of Low Frequency Magnetic Field LFMF Square Magnetic Pulses SMP on *P. mirabilis* ATCC 9240 and to find out the resonance frequency that can inhibit its activity and its ability to make division. Moreover, to investigate the changes that may occur on the viability, antibiotic susceptibility dielectric characteristic and morphological structure.

2.MATERIAL AND METHODS

The *P. mirabilis* strain (ATCC 9240) used in this study was purchased from the Holding Company for Biological Products and Vaccines (VACSERA) in Egypt.

A broth subculture Prepared by inoculating a test tube containing 7 ml of sterile nutrient broth media of pH 7.1 (Biolife, Milan, Italy) with two colonies of bacteria from nutrient agar plate, then incubated at 37°C for 24 h.

With this subculture, 500-ml screw-capped flask containing 150 ml of sterile nutrient broth media was inoculated to reach a final concentration of 10^7 cfu ml^{-1}. Then the cultures were incubated at 37°C, but the incubation was interrupted each 2 h, as the sample was taken for measuring the absorbance (using sterile broth media as reference) at wavelength 600 nm using a spectrophotometer (6405 UV/Vis; Jenway, Stone, Staffordshire UK). Each experiment was executed in triplicates and the average was considered. The viable plate counting technique **(Skinner et al., 1952)** carried out to determine the concentration of bacterial cells (cfu ml^{-1}) in parallel with the absorbance measurements. A standard count-absorbance calibration curve was plotted between the absorbance of the samples at 600 nm and the concentration of the cells (cfu ml^{-1}) as determined by plate counting technique **(Atlas 1995)**.

SMP Exposure System

The SMP was generated inside a locally made hollow cylindrical copper solenoid of 11 cm in diameter and 16 cm in length, manufactured at the Electronic workshop in the Faculty of Science, Cairo University.

The solenoid was constructed electrically insulated copper wire coils wounded around the outer surface of an electrically insulated copper cylinder. The apparatus generates 50 milligauss magnetic flux density. The homogeneity of the magnetic field inside the solenoid was measured at different locations by using a Gauss/Tesla meter (Model 4048 with probe T-4048.001 by Bell Technologies, Inc., USA) of accuracy ±2%. As shown in **figure (1)**.

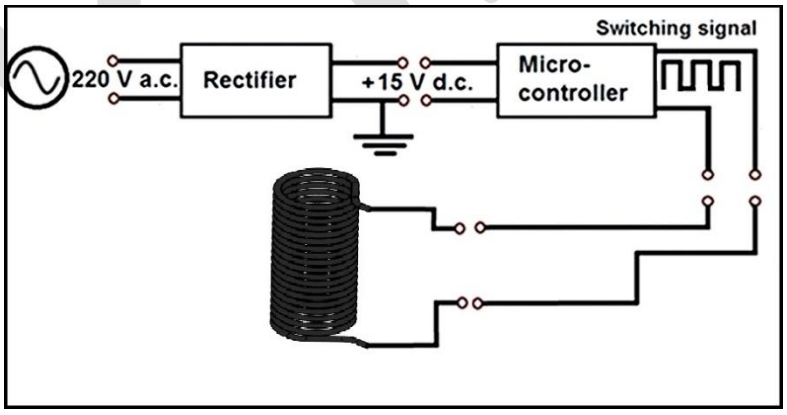

Figure (1): Schematic illustration of the experimental design used for exposure.

Inhibition Frequency Determination:

A broth subculture was prepared by inoculating a test tube containing 7 ml of sterile nutrient broth of pH 7.1 (Biolife, Milan, Italy) with two colonies of bacteria from nutrient agar plate, followed by incubation at 37°C for 24 h.

With this subculture, 12 test tubes containing 7 ml of sterile nutrient broth were divided into 4 groups, one as a control and the others were exposed to SMP in the range from 0.5 to 0.7 Hz in steps of 0.1 Hz for 30 minutes in order to determine the resonance frequency of growth inhibition at which the peak of the inhibition is occurring and during the exposure period all the groups were kept at the room temperature. After the exposure period, all the groups (control and exposed) were then incubated at 37°C, but the incubation was interrupted each two hours, as samples were taken for absorbance measurement (using sterile broth media as reference) at wavelength 600 nm using a spectrophotometer. The inhibition percentage difference at 8^{th} hour incubation for each sample is calculated with respect to the control according to equation (1) and the average value is taken. Then the average inhibition percentage difference values are plotted versus frequency in the range of 0.5 Hz to 0.7 Hz to represent the resonance curve for the analysis purpose.

D% = [(Average OD (control) – Average OD (exposed)) / Average OD (control)] x 100. (1).

The previous experiment was repeated with all the same conditions, but at exposure period 60 minutes. Growth curves were plotted and the inhibition percentage difference at 8^{th} hour incubation for each sample is calculated with respect to the control.

Antibiotic susceptibility test

The bacterial isolate of *P. mirabilis* was subjected to susceptibility testing using eight different antimicrobial agents, namely: Amikacin (AK), Norfloxacin (NOR), Levolfloxacin (LEV), Cefepime (FEP), Cefoperazone (CEP), Piperacillin/ Tazobactam (TPZ), Amoxicillin/ Clavulanic(AMC), S-Adenosylmethionine (SAM)). The susceptibility of *P. mirabilis* against these antibiotics was measured by disc diffusion method, which was carried out and performed by the procedure supplied by CLSI, M100-S25 (clinical and laboratory Standards institute for Antimicrobial Susceptibility Testing; 2015). *P. mirabilis* suspension was divided into three groups (three samples per each); control and exposed to SMP at resonance frequencies for exposure times 30 and 60 minutes. After the exposure periods, samples of control and exposed groups were inoculated in MacConkey agar plates. After the inoculants had dried (3-5 minutes), the proper antibiotic discs were placed on the agar surface with a sterile forceps, gentle pressure was applied over the surface of each disc to ensure contact. The inoculated plates, then incubated at 37 °C for 24 hours. Then the diameter of each inhibition zone was measured and compared with the zone reading chart.

Dielectric measurement

The dielectric measurements have been carried out for control and the exposed samples to SMP at resonance frequency and the most effective inhibition time. The experiment was carried out in the frequency range 1.0 Hz – 100 KHz using a Loss Factor Meter (HIOKI 3532 LCR Hi TESTER, version 1.02, 1999, Japan), with a sample cell (PW 9510/60, manufactured by Philips, Japan). The sample cell is made of glass, has two squared platinum black electrodes of 0.64 cm^2 area and separated by 1 cm apart.

The temperature throughout the experiment was kept constant at 25±0.1^{o} C. The cell was immersed in a glass beaker containing diluted bacterial sample suspension in which the suspension covers the entire volume between the cell electrodes. The capacitance of the samples was measured at each frequency and the resistance was recorded. Each run was taken three times and the average was considered.

The value of relative permittivity (έ), loss tangent (tan δ), dielectric loss (ε″), conductivity (S) and relaxation time (τ) of the samples were calculated through the following equations.

$\acute{\varepsilon} = Cd / \varepsilon_O A$ (2)

Where ε_O is permittivity of free space.

$\tan\delta = 1/2\pi FRC = \varepsilon''/\acute{\varepsilon}$ (3)

$\sigma = 2\pi F\varepsilon''\varepsilon_O$ (4)

$\tau = 1 / 2\pi F_c$ (5)

Where F_c is the frequency at the midpoint of the dielectric dispersion curve.

$S = \sigma / \varepsilon_O = 2\pi F\acute{\varepsilon}$ (6)

This has the advantage that S is measured in s^{-1} rather than $Ohm^{-1}\ m^{-1}$, which is the unit of σ.

Transmission Electron Microscope (TEM) Examination:

The morphological changes of control and exposed samples to SMP at 0.6 Hz for (60 minutes) have been examined using TEM (JEM-1400; JEOL Ltd., Akishima, Tokyo, Japan) provided with CCD camera model AMT, optronics camera with 1632 x 1632 pixel existed in the Faculty of Agriculture Research Park-Cairo University. The microbial cells subjected to some processing **(Rocchetta et al., 2007)** for the preparation to TEM imaging. The processing of bacterial cells (control and exposed) was carried out after one hour of exposure to SMP.

3.RESULTS

Growth Characteristics

At 600 nm, the optical density of *P. mirabilis* sample variance with cell count in nutrient broth medium showed a linear dependence of the absorbance on the micro-organism count with a slope $N/A=1.29x10^{8}$; where N is the number of micro-organisms per milliliter and A is the absorbance of the sample.The growth curves were plotted for different samples after the exposure to SMP for a period of 30 min at different frequencies in order to compare with the growth curve of the control sample. The results indicated variation of inhibition in the absorbance of the sample exposed to 0.6 Hz as shown in **figure (2)**. The change of absorbance at the 8th hour after incubation was measured and the results in **figure (3)** showed the inhibition resonance peak for cellular growth at frequency 0.6Hz.

In this regard, the number of CFU/ml for the control and exposed samples at those resonance frequencies after 8th hour incubation are tabulated in **table (1)**.

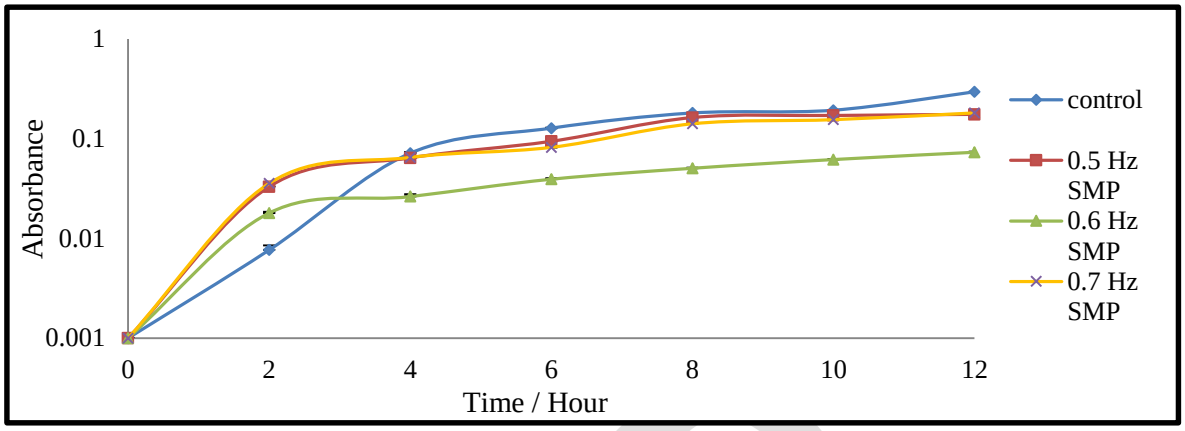

Figure (2): The growth curve of *P. mirabilis* exposed to different frequencies SMP for a period of 30 minutes.

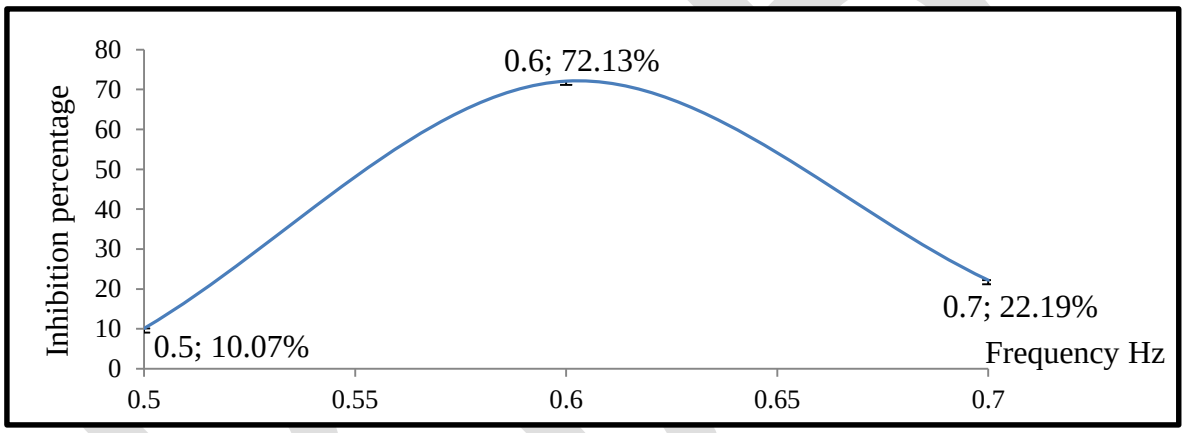

Figure (3): Inhibition percentage of the exposed samples with respect to control at different frequencies SMP and fixed time 30 minutes (at the 8th hour of incubation).

Table (1): The cell count in CFU/ml for control and exposed Samples to SMP for 30 minutes with its inhibition percentage difference.

Sample / Exposure frequency	30 minutes SMP		
	0.5 Hz	0.6 Hz	0.7 Hz
Control (*10^5 CFU/ml)	234.35±2.43		
Exposed (*10^5 CFU/ml)	210.7±2.19	65.36±1.6	182.32±0.61
Real count in plates (*10^5 CFU/ml)	205.23±3.14	47.31±2.13	167.3±1.92
Inhibition percentage difference D%	(10.07%)***	(72.13%)***	(22.19%)***

NS Not Significant *Significant (P<0.05) **Highly Significant (P <0.01) ***Very Highly Significant (P <0.001).

It is obvious from the table that the growth characteristics of samples exposed to SMP have been reduced by 10.07%, 72.13% and 22.19% at frequencies 0.5Hz, 0.6Hz and 0.8 Hz respectively.

Then, the growth curves were plotted for different samples exposed to SMP at different frequencies in ranges from (0.5 to 0.7) Hz in steps of 0.1 Hz for a period of 60 minutes. The results indicated variation of inhibition in the absorbance of the sample exposed to 0.6 Hz as shown in **figure (4)**.

Hence, the change of absorbance at 8th hour after incubation was measured and the results in **figure (5)** showed the inhibition resonance peak for cellular growth at frequency 0.6Hz.

In this regard, the number of CFU/ml for the control and exposed samples at those resonance frequencies after 8th hour of incubation are tabulated in **table (2).**
It is obvious from the table that the growth characteristic of samples exposed to SMP has been reduced by 34.8%, 74.42% and 25.6% at frequencies 0.5Hz, 0.6Hz and 0.7Hz respectively.

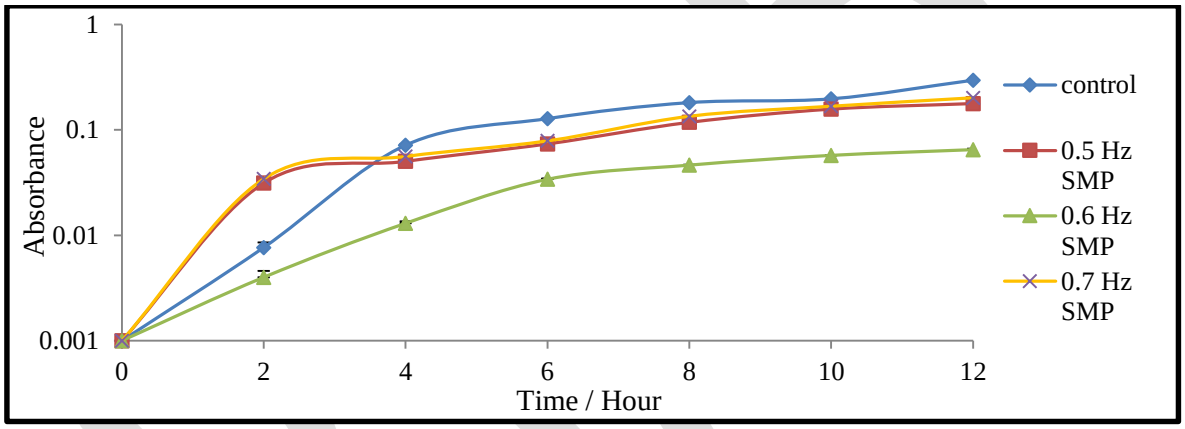

Figure (4) :The growth curve of *P. mirabilis* exposed to different frequencies SMP for a period of 60 minutes.

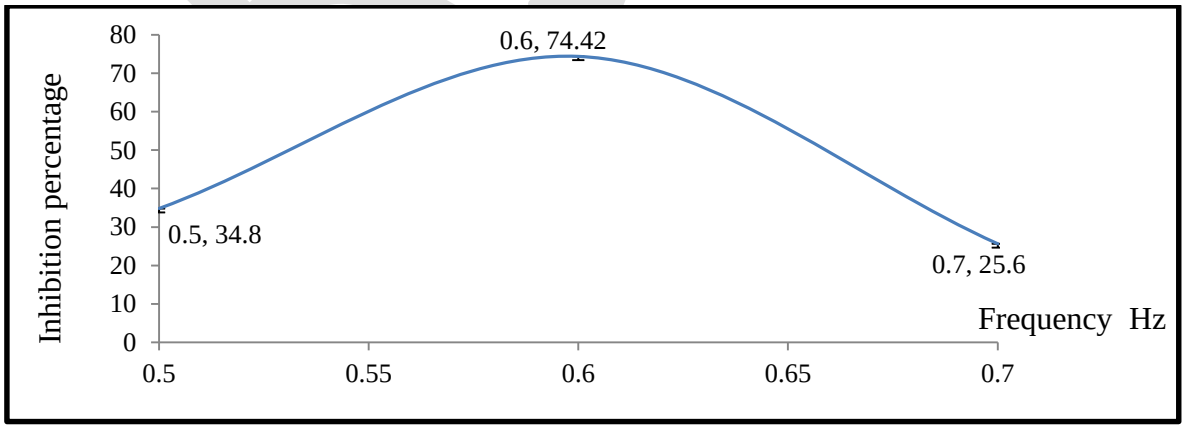

Figure (5): Inhibition percentage of the exposed samples with respect to control at different frequencies SMP and fixed time 60min (at the 8th hour of incubation).

Table (2): The cell count in CFU/ml for control and exposed Samples to SMP for 60 minutes with its inhibition percentage difference.

Sample / Exposure frequency	60 min SMP		
	0.5 Hz	0.6 Hz	0.7 Hz
Control (*10^5 CFU/ml)	234.35±2.43		
Exposed (*10^5 CFU/ml)	152.22±1.05	59.77±0.61	173.72±0.61
Real count in plates (*10^5 CFU/ml)	147.61±1.24	48.34±1.43	168.21±2.17
Inhibition percentage difference D%	(34.8%)***	(74.42%)***	(25.6%)***

NS Not Significant *Significant (P<0.05) **Highly Significant (P <0.01) ***Very Highly Significant (P <0.001).

The antibiotic susceptibility test

The antibiotic susceptibility test results for control and exposed samples to 0.6 Hz for 30 and 60 minutes are given in the table (3-a & 3-b) showing a significant difference between the control and exposed samples.

It is obvious from the table that, samples exposed to SMP at 0.6 Hz gave a significant increase in susceptibility to protein inhibitors (AK), DNA inhibitors (NOR and LEV) cell wall synthesis inhibitor (FEP and CEP), β-lactamase enzyme and cell-wall synthesis Inhibitors (AMC and TPZ) and DNA and RNA nucleic acids, phospholipids, proteins Inhibitors (SAM).

Table (3-a): The mean inhibition zone diameter (mm) of different antibiotic agents for the control and exposed *P. mirabilis* samples.

Antibiotic / Sample		Mean Inhibition Zone Diameter in mm			
		Protein Inhibitors	DNA inhibitors		(DNA and RNA nucleic acids, phospholipids, proteins) Inhibitors
		AK(30mcg)	NOR(10mcg)	LEV(5mcg)	SAM(20mcg)
Control (unexposed)		(13±0.8)	(34±0.47)	(35±0.47)	0
0.6 Hz	30 minutes SMP	(18±0.47)***	(37±0.8)**	(37±0.94)**	(9±0.8)***
	One Hour SMP	(28±0.8)***	(39±0.47)***	(40±0.8)***	(22±0.47)***

Table (3-b): The mean inhibition zone diameter (mm) of different antibiotic agents for the control and exposed *P. mirabilis* samples.

Antibiotic / Sample	Mean Inhibition Zone Diameter in mm			
	Cell Wall Inhibitors		β-lactamase enzyme and cell-wall synthesis Inhibitors	
	FEP(30mcg)	CEP(75mcg)	AMC(30mcg)	TPZ(110mcg)

Control (unexposed)		(12±0.47)	(14±0.8)	0	(17±0.47)
0.6 Hz	30 minutes SMP	(14±0.8)*	(15±0.47)NS	(8±0.47)***	(22±0.8)***
	One Hour SMP	(21±0.47)***	(25±0.47)***	(13±0.8)***	(29±0.8)***

NS Not Significant *Significant (P<0.05) **Highly Significant (P <0.01) ***Very Highly Significant (P <0.001).

The dielectric measurement

Figures (6,7) show the dielectric dispersion, which relates dielectric constant (έ) and conductivity (S) as a function of frequency. The figures show the effects on the molecular structure where they demonstrate the variation between the control and exposed samples at the resonance frequency and the most effective inhibition time. It is obvious from the figures that, the dependence of (έ) on the frequency make a mirror image with the dependence of (S) on the frequency, where any decrease in the permittivity is accompanied by increase in the conductivity. This yields a confirmatory test for data **(Foster and Schwan 1989)**.

Dielectric increment ($\Delta\acute{\varepsilon} = \acute{\varepsilon}_\infty - \acute{\varepsilon}_o$), the relaxation time (τ), and conductivity (S) were calculated from the curves in the figures for all samples and The average values of each are calculated in the **table (4).**

The results indicated that, there is a significant increase of the dielectric increment (Δέ), conductivity (S) and the relaxation time (τ) for the exposed samples as compared with the control.

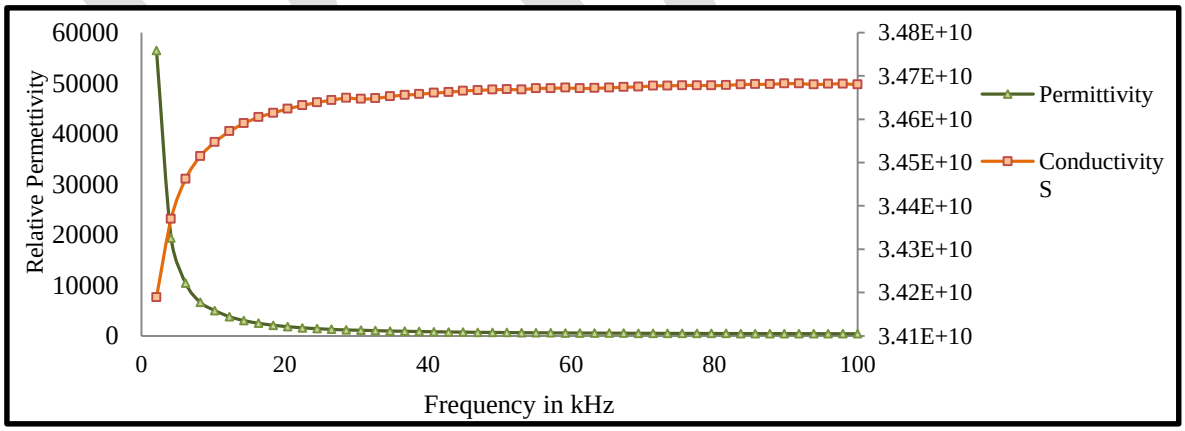

Figure (6): The variations of the relative permeability (ε') and electric conductivity (σ) as function of frequency in the range 0.1-100kHz for control sample bacterial suspension.

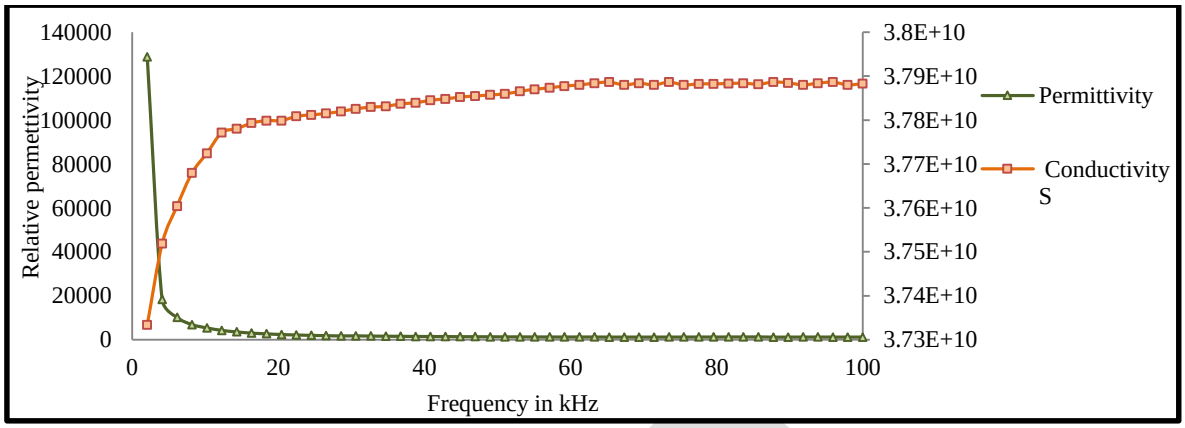

Figure (7): The variations of the relative permittivity (ε') and electric conductivity (σ) as a function of frequency in the range 0.1-100kHz for sample bacterial suspension exposed to 0.6 SMF for one hour.

Table (4): The relaxation time (τ), the dielectric increment (Δέ) and the conductivity (S) of control and exposed samples to 0.6Hz SMP for one hour.

Sample	τ (ms)	Δ έ	Conductivity (S) *10^8
Control	(0.45±0.012)	(56040.84±576.9)	(341±54.4)
One Hour SMP	(0.81±0.007)***	(128970.9±545.8)*	(373.3±34.1)*

NS Not Significant *Significant (P<0.05) **Highly Significant (P <0.01) ***Very Highly Significant (P <0.001).

Transmission Electron Microscope (TEM) images:

Figures (8-a) shows TEM images obtained from control and **figure (8-b)** shows TEM images obtained from exposed samples to SMP under experimental conditions one hour exposure, which reveal changes.

Observations on the ability of SMP to induce changes of cell wall and the internal inclusions were clearly noticed **in figure (8-b)** where flagella were absent or lost after exposure of *P. mirabilis*, which are possessing peritrichous flagella and is known for its swarming ability, to SMP for one hour. Meanwhile, the cell wall shape seemed to be affected or damaged and that may make interruption in the metabolic activity like stop secreting urease enzyme which is hydrolyzing urea into ammonia and carbon dioxide resulting in increasing in pH forming urinary stones and inflammations, in addition to a less dense electron space and heterogeneous appearance of the cytoplasm.

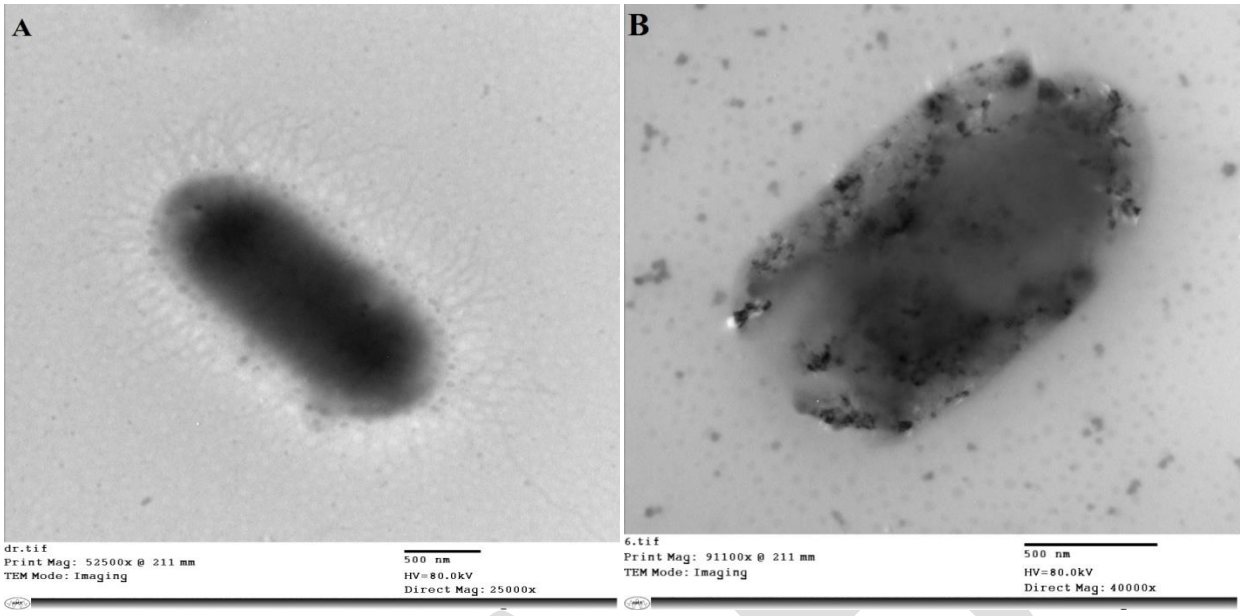

Figure (8): TEM images for the control sample (a), samples the exposed to 0.6 Hz SMP (b).

4.DISCUSSION

In this work; the resonance frequency of SMP that inhibits the cellular division of *P. mirabilis* was determined. It is well known that the metabolic mechanisms of living biological systems run through ionic motion. The rate of movement of these ionic charges forms ionic currents resulted in ionic potentials. The wave form and frequency of these bioelectric potentials represent the running physiological process which can be understood as the fingerprint of the system. These bioelectric currents generate loops of bio-magnetic fields which disturb the metabolic functions of the neighboring cells.

Based on the Metabolic Bio-magnetic Resonance Model (BMRM), suggested by Fadel **(Ali 1998)**, an external applied electromagnetic signal can interfere with a bioelectric signal when they are at resonance. The resultant of the interference is the algebraic summation of the two waves which may be instructive or destructive, i.e. enhancement or inhibition for the running process **(Ali et al., 2010)**. Based on these bases the present work was planned.

From the basic understanding and Wright hand rule, when an external magnetic field is applied on moving ions, a magnetic force is generated on the ions perpendicular to their flow direction and the applied magnetic field. This magnetic force will shift the direction of the moving ions to circular pathway. The shift of these ions from their targeted direction will distort the running physiological process.

Recent studies **(Ali et al., 2011; (Ali et al., 2003)** demonstrated that exposure to ELF magnetic fields had a significant influence on the packing properties of the phospholipids macromolecules forming the cellular membrane and can cause changes in the Van der Waals forces binding these molecules together to form the membrane. These changes in the membrane mechanical properties lead to the membrane permeability alteration which can be a reason for the observed destruction of the microbial cellular

membrane noticed in the TEM images for the treated microbe by SMP.

The present analyses were born out from the results of the TEM images where, the disturbance of the macromolecules binding forces may result in the breakage noticed in the cell wall of the bacteria and hence the possible flow of the surrounding media to cross inside the cell and can cause changes in the DNA properties. This finding is supported by the significant increase of the electrical conductivity of the bacterial culture for the irradiated sample. Furthermore, the increase of the electrical conductivity of the exposed sample compared to the control, indicates the increase in the surface electric charge of the wall of the bacteria. Which may lead to the increase in the dipole moment of the macromolecules forming the cell wall associated with an increase in the dielectric increment and also disturbance in the cell-cell communication. All these effects may be the main reason for the inhibition of the cellular growth after irradiation. This discussion is supported by the TEM images and the significant increase in the antibiotic sensitivity test for protein, DNA and cell wall synthesis inhibitors.

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