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EFFECT OF SOME FATTY ACIDS ON VIRULENCE FACTORS OF *PROTEUS MIRABILIS*

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ABSTRACT
A total of 131 urine samples from catheterized urinary tract for patients in intensive care units were collected from five hospitals at Baghdad over three months. Urine samples were cultured on blood agar & Mac Conkey medium; 103 sample were positive bacteriological culture (78.6%); the isolates have been diagnosed depending on morphological characters & biochemical tests, *Proteus spp.* 22 isolate (21.3%) were diagnosed by using vitek 2 Gram Negative Identification (GNI) cards; *Proteus mirabilis* isolates were (17) with percentage (77%), while *Proteus vulgaris* were (5) with percentage (23%). The sensitivity of the isolates against (14) antibiotics was investigated, the results showed high resistance of the isolates to many antibiotics especially for erythromycin (100%) while they were sensitive to imipenem (100%) followed by nitrofurantien (94%) then ciprofloxacin (82.3 %). Multiple concentrations of fatty acids (lauric, palmitic, oleic, stearic) have been prepared & tested on some virulence factors (swarming, heamolysin, biofilm formation) of *P. mirabilis* isolates as well as the effect of fatty acids on the content of sugar & fat of lipopolysaccharide have been examined. The results indicated that the lauric acid was the most effective fatty acid in the inhibition of virulence factors while oleic acid was good stimulant for production of heamolysin & swarming, also there was noticeable inhibition of biofilm formation by lauric & oleic acids, as well as the results showed that all fatty acids in this study have the ability to decrease the content of sugar & fat of lipopolysaccharide.

KEYWORDS: MacConkey medium, *Proteus mirabilis*, lipopolysaccharide, fatty acids.

INTRODUCTION

Urinary tract infections (UTIs) is one of the most common hospital-associated infections[1]. Placement of an indwelling catheter predisposes individuals to the development of catheter-associated (CaUTI), the most common type of nosocomial infections[2,3]. (CaUTI) is generally thought to be caused by self-inoculation of the catheter. Once bacteria have colonized the catheter, motile species can rapidly transverse the catheter surface to reach the bladder and potentially establish a UTI[4]. The dimorphic, motile, gram-negative bacterium *P. mirabilis* is one of the leading causative agents of CaUTI, responsible for up to 44% of these infections[3]. *P. mirabilis* infections frequently develop into cystitis and pyelonephritis and can be further complicated by catheter encrustation and formation of urinary stones[5]. The ability of *P. mirabilis* to express virulence factors, including urease, protease, and hemolysin and to invade human urothelial cells is coordinately regulated with swarming differentiation[6]. Fatty acids or their derivatives have been shown to be involved in the regulation of swarming differentiation and virulence factors expression[7]. Effect of fatty acids on virulence factors of *P. mirabilis* have been studied rarely[8], so this study was aimed to investigate the role of some fatty acids in inhibition the virulence factors of *P. mirabilis*.

MATERIALS & METHODS

Materials

131 Urine samples were collected from catheterized patients at intensive care units from 5 hospitals/ Baghdad. 103 were positive bacteriological culture. Bacteria; *P. mirabilis* isolates 17 have been diagnosed by morphological & biochemical tests, the diagnosis was confirmed by Vitek 2 system provided by BioMereurix (France).
Standard strain: *Escherichia coli* 25922 was provided from central health lab. Fatty acids & chemicals; were provided by B.D.H (England), Fluka (Germany).
Culture media: Oxoid (England), Himedia (India) with the exception of Luria-Bertonia (agar & broth) & Casamino acid-Pepton-Glucose broth were prepared as structural media. Antibiotics discs were provided by Bioanalyse (Turkey). Reagents & solutions; were prepared in the microbiology lab. Biology department, College of Science, Al-Mustansiriya University.

METHODS

1- Fatty acids concentrations have been prepared according to [9] as following;
Four types of fatty acids have been tested in this study; Oleic, stearic, palmitic, lauric acid; Saturated fatty acids: stearic, palmitic, lauric; 0.01, 0.005, 0.0025 gm/each fatty acid by the equation:

$$W/V\% = \frac{Wt.\ of\ solute}{V.\ of\ solvent} \times 100$$

Non saturated: oleic acid; 2.2 ml dissolved in 96 % absolute alcohol then added D.W to 100 ml conc. was calculated as in equation;

$$V/V\% = \frac{V.\ of\ solute}{V.\ of\ solution} \times 100$$

Wt =weight
V = volume

Mixing of acids with the culture media was performed as following;
Calculation of medium &prepared acid weight *i.e.*, if the medium weight was 4gr dissolved in 100ml, acid weight was 0.01 gm:

100	4
X	0.01

x=0.25 ml of acid to get 0.01gr/ml

If the tested conc. = 0.005 gr/ml, acid added was 0.12 ml, 0.0025gr/ml ---0.066ml.
If the medium is broth, the acid added was 0.12, 0.066, 0.033 ml respectively.

2- Antibiotic sensitivity test have been carried out according to[10] using Kirby-bauer disc diffusion method.
3- The isolates (1, 3, 8, 9, 13) which were the most resistant to antibiotics, have been chosen for the studying the influence of fatty acids on virulence factors.
4- All the experiments; swarming, heamolysin activity, biofilm formation, LPS content have been tested on the selective medium (LB agar & broth) + fatty acid (4 types with 3 concentrations) as well as the control (agar & broth without fatty acid), these tests have been repeated 3 times for each isolate.
5- No. of the bacterial cell in each experiment is constant (1.5×10 cell/ml).
6- Bacterial isolates were tested for their ability to form biofilms according to[11], flat bottomed 96 well microtiter plates were inoculated with 200µl of bacterial suspension corresponding to 0.5 McFarland and incubated at 37ºC for 24 hrs, contents were removed by decantation, washing 3 times, fixing by hot air, staining by crystal violet, washing, adding ethanol.
7- Effect of fatty acids on LPS extraction have been tested according to[12] after culturing the isolates with &without fatty acids on LB broth, shaking incubation overnight, centrifugation, sediment have been washed twice, suspended in CPG broth, centrifugation, suspension of supernatant with ethanol, centrifugation, sediment was suspended, mixing then centrifugation, supernatant + ethanol centrifugation, sediment + sterilized D.W.

*Carbohydrates in LPS were estimated by drawing standard curve of different concentrations of glucose.
*Quantity of carbohydrates has been estimated by phenol-sulphuric acid according to[13]. Results were determined by spectrophotometer at 490 nanometer.
*Lipids of LPS were estimated according to[14], concentrated sulphuric acid & vanillin solution. Optical density has been read at wave length 595 nanometer.
8- **Statistical analysis:** results were converted to computerized database then processed by using statistical Package for social Sciences Version 21.

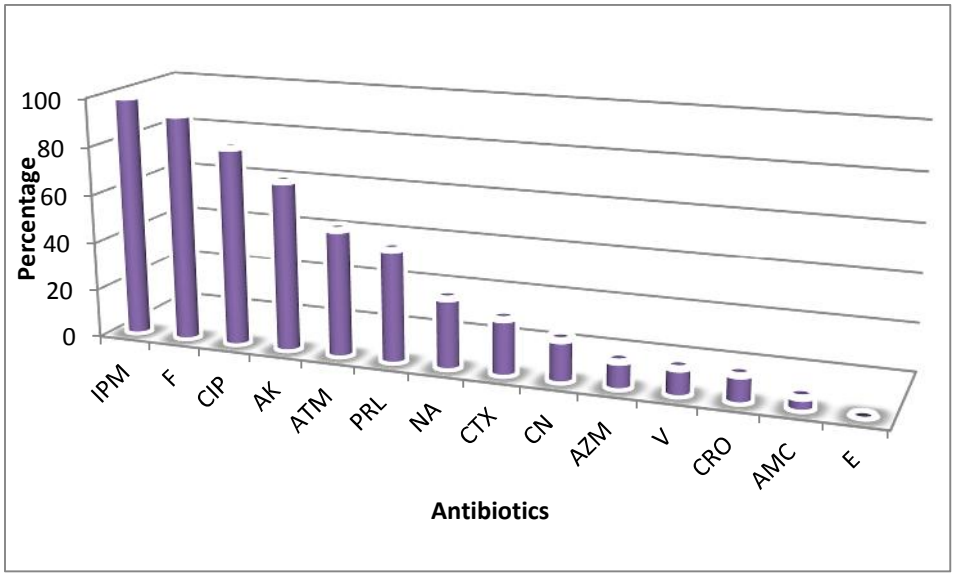
RESULTS & DISCUSSION

Prevalence of *Proteus spp.* has been detected in catheters of UTIs patients of both genders & all ages. Percentage of isolation (21.3%) was agreed with the results[15] which were (19.6%), while the percentage of[16] about UTIs in children at Australia was (5%), differences may be appeared because of different ages, technician errors, varieties from region to another.
Isolates percentage of *P. mirabilis* was (77%) while *P. vulgaris* was (23%), this have been agreed with the results of [17] that was (81.96); this may be attributed to high prevalence of this *P. mirabilis* in human stool comparable to *P. vulgaris*.
Identification of *Proteus spp.* have been carried out depending on morphological characters of colonies on blood agar which have very characteristic & distinguishable phenomena (swarming) & fishy odor, as well as colonies were pale on macConkey agar-non-fermentor for lactose[6], also *Proteus spp.* produce urease enzyme, and FeS on KIA, gram-negative short bacilli staining red to pink[18]. Vitek 2 diagnostic kit have been used for confirmation the identification of results, GN ID (Card) of vitek 2 compact is accurate & rapid, this system have new & additional tests. 47 tests were performed at 6 hours, diagnosis short time prevent mutation to occur, high accuracy, no false results as well as it providing identification percentage as in appendix (1).
Results of antibiotic sensitivity were showed high resistant of *P. mirabilis* for almost antibiotics used in the studyas demonstrated in appendix (2). Isolates showed high resistance (94.2%) to augmentin (penicillins), (76.5%) for cefotaxime & (88.3%) for ceftriaxone (third generation of cephalosporins), intermediate resistance was appeared for aztreoname (47.1%) of monobactam that may be refers to the ability of these bacteria to producing Extended Spectrum Beta Lactamase (ESBLs). Study have been shown high resistance (82.4%) to gentamycin which represent aminoglycoside group while isolates were sensitive to amikacin with percentage (29.5%), good resistance of bacteria to nalidixic acid (quinolones) have been reported (70.6%).Resistance for this antibiotic may result from genetic mutation in the active site as well as inhibition the DNA gyrase that responsible for strand coiling that would provides bacteria this high resistance[19].

APPENDIX 1: Biochemical Tests of the Card of *P. Mirabilis* (Vitek 2)

Test	Symbol	Hole No.	Test	Symbol	Hole No.
SACCHARIDE –SUCROSE	SAC	33	Ala-phe-pro-ARYLAMIDASE	APPA	2
D- TAGATOSE	dTAG	34	ADONTOL	ADO	3
D- TREHALOSE	dTRE	35	L-PYRROLYDONYL-ARYLAMIDASE	PyrA	4
CITRATE(SODIUM)	CIT	36	L-ARABITOL	IARL	5
MALONATE	MNT	37	D-CELLOBIOSE	dCEL	7
5- KETO- D- GLUCONATE	5KG	39	BETA-GALACTOSIDASE	BGAL	9
L- LACTATE-ALKALINISATION	ILATK	40	H2S PRODUCTION	H2S	10
ALPHA- GLUCOSIDASE	AGLU	41	BETA-N-ACETYLGLUCOS AMINDASE	BNAG	11
SUCCINATE-ALKALINSATION	SUCT	42	GLYTAMYL ARYLAMIDASE	AGLTp	12
	NAGA	43	D- GLUCOSE	dGLU	13
ALPHA-GALACTOSIDASE	AGAL	44	GAMMA-GLUTANYL-TRANSFERASE	GGT	14
PHOSPHATASE	PHOS	45	FERMENTATION GLUCOSE	OFF	15
GLYCINE ARYLAMIDASE	GlyA	46	BETA-GLUCOSIDASE	BGLU	17
ORNITHINE DECARBOXYLASE	ODC	47	D- MALTOSE	dMAL	18
LYSINEDE	LDC	48	D- MANNITOL	dMAN	19
L- HISTIDINE ASSIMILATION	IHISa	53	D- MANNOSE	dMNE	20
COUMARATE	CMT	56	BETA- XYLOSIDASE	BXYL	21
BETA-GLUCORONIDASE	BGUR	57	BETA-ALANINE ARYLAMIDASE	BAIap	22
O129- RESISTANCE	O129R	58	L- PROLINE ARYLAMIDASE	Pro A	23
GLU-GLY-ARG-ARYLAMIDASE	GGAA	59	LIPASE	LIP	26
L-MALATE ASSIMILATION	IMLTa	61	PALATINOSE	PLE	27
ELLMAN	ELLM	62	TYROSIN ARYLAMIDASE	TyrA	29
L-LACTATE ASSIMILATION	ILATa	64	UREASE	URE	31
			D- SORBITOL	dSOR	32

Bacterial isolates were sensitive to flouroquinolones, percentage of resistance was (17.7%) for ciprofloxacin its higher than percentage of [20,21], which were (3.7%) & (6.5%) respectively, but was agree with the results of[15], these are some of local studies while the global studies of [22,23] reported the percentages of resistance (24.5%), (23%) respectively. Ciprofloxacin is one of broad spectrum antibiotics in their effect on pathogenic bacteria; recently it is the most important drug for UTIs treatment[24]. Enteric bacteria have different mechanisms enable them to resist quinolones & flouroquinolones like genetic mutations that code for new DNA gyrase & topoisomerase[25], resistance also may attributed to impermeable cell membrane & changing the porins leading to decrease the antibiotic accumulation inside bacterial cell[26]. Antibiotic sensitivity results showed high resistant to vancomycin & erythromycin, the percentages (88.3%), (100%) respectively, these results were agreed with the study of[27], she documented high sensitivity(100%) of *P. mirabilis* isolated from burns & wounds at Baghdad city, also the bacterial isolates were highly sensitive to nitrofurantoin (5.9%). Figure (1) demonstrates the percentages of sensitivity of *P. mirabilis.*



Ipm = Imipenem , F = Nitrofurantien , Cip = Ciprofloxacin , Ak = Amikacin ATM = Aztroename , PRL = Piperacillin , NA = Nalidixic acid , CTX = Cefotaxime , CN = Gentamycin , AZM = Azithromycin , V = Vancomycin , CRO = Ceftriaxone , Amc= Amoxicilln-clavulonic acid , E = Erythromycin

FIGURE 1: Percentages of *P. mirabilis* Isolates Sensitivity against antibiotics

APPENDIX -2: Sensitivity of towards Antibiotics

Antibiotic	Symbol	Conc. µg/disc	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Pipracillin	PRL	30	S	R	S	S	R	R	S	R	R	R	S	R	S	R	S	R	S
Cefatoxime	CTX	30	R	R	R	S	S	R	R	R	R	R	R	R	R	R	S	R	S
Imipeneam	IPM	10	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
Gentamycin	CN	10	S	R	R	R	S	R	R	R	R	R	S	R	R	R	R	R	R
Ciprofloxacin	CIP	10	S	S	S	S	S	R	R	S	R	S	S	S	S	S	S	S	S
Naldixic acid	NA	30	S	R	R	R	S	R	R	R	R	S	R	R	R	R	R	S	S
Aztreoname	ATM	30	R	S	R	S	R	R	R	S	R	S	R	S	R	S	S	S	S
Azithromycin	AZM	15	R	R	R	S	R	R	S	R	R	R	R	R	R	R	R	R	R
Amikacin	AK	10	S	S	R	S	S	S	R	S	R	S	R	S	R	S	S	S	S
Nitrofurantoin	F	300	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S
Cefatrixone	CRO	30	R	R	R	R	S	R	R	R	R	S	R	R	R	R	R	R	R
Amoxicillin_ clavulonic acid	AMC	30	R	R	R	R	S	R	R	R	R	R	R	R	R	R	R	R	R
Vancomycin	VA	10	R	R	R	R	R	R	R	R	R	R	R	S	R	R	S	R	R
Erythromycin	E	15	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

*S = Sensitive *R= Resistant

Effect of fatty acids on swarming

Role of fatty acids in inhibition of swarming have been investigated because of the importance of this phenomenon as a virulence factor of pathogenic bacteria, swarming help bacterial cell to move & transport leading to host penetration, swarming also increase virulence by encoding to producing hemolysins & flagellin (29). Fatty acids have been added in different concentrations (0.01%, 0.005%, 0.0025%) to the inoculating culture media, figures 2,3, 4 demonstrate the results:

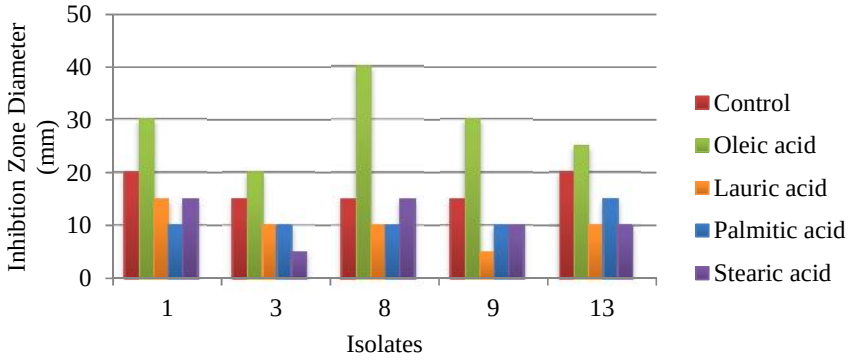


FIGURE 2: Effect of Fatty Acids (0.01%) on Swarming

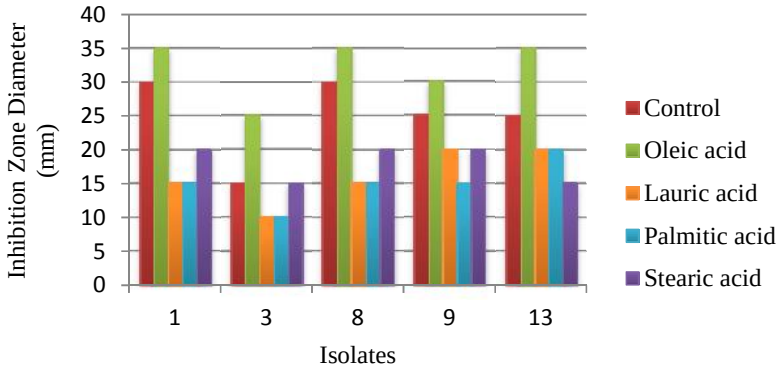


FIGURE 3: Effect of Fatty Acids (0.005%) on Swarming

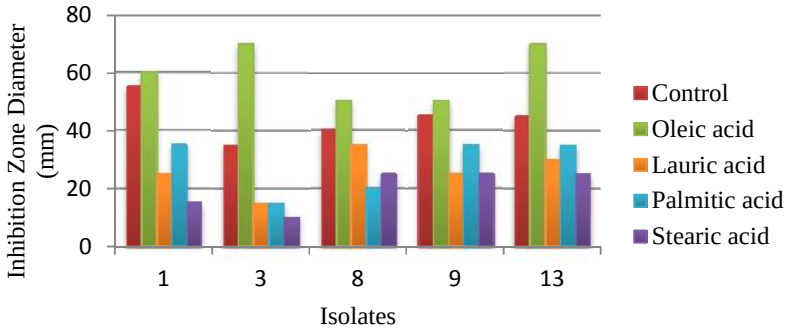


FIGURE 4: Effect of Fatty Acids (0.0025%) on Swarming

The results have been clarified the ability of lauric, palmitic & stearic acids on inhibition swarming compared with control plates (without fatty acid), while the fatty acid; oleic had been encourage & support swarming occurring specially with the isolate no. (3) as shown in figures. Statistical analysis appeared high significant differences at level (P≥0.05). These results were agreed the results of [8] who reported encouragement & promotion for swarming to occurring by the oleic fatty acid that increase the diameter (5-7cm). Rare Iraqi studies are concern with this subject so we compared our results with the effect of another materials & compounds on swarming like ammonium potassium sulfate[29], also EDTA, sucrose, sodium chloride, sodium acetate 80, phenol[30], as well as different concentrations of amikacin in inhibition of swarming [31].

Effect of Fatty Acids on Heamolysin Activity

Heamolysins are important virulence factors that stimulate pathogenicity, the most effective type is α-heamolysin, which cause skin necrosis & hemolysis, table (1) demonstrate the types of heamolysins that produced by *P. mirabilis* isolates:

TABLE 1: Types of Heamolysis Produced by *P. mirabilis*

Type	Isolates No.	(%)
Non-producer	2	11.7
[illegible]	6	35.3
[illegible]	9	53.0
Total	17	100

Where α (35.3%) means partial heamolysis, and β (53%) means complete heamolysis.

Detection for enzyme activity at different temperatures (25°C,37°C,42°C) have been carried out in LB broth treated & untreated with the four types of fatty acids, LB broth was inoculated with five tested isolates (1,3,8,9,13). SDS was used for complete heamolysis (control). Results showed that the higher rate of enzyme production at 37 °C that is the optimum temp. for production & activity of most enzymes, any increase more than 50◦C results in enzyme denaturation[32]. Lauric fatty acid have clear inhibitory effect on heamolysin activity with three concentrations at three temperatures, while oleic acid has been enhanced the production & activity of heamolysin, results were shown in figures below:

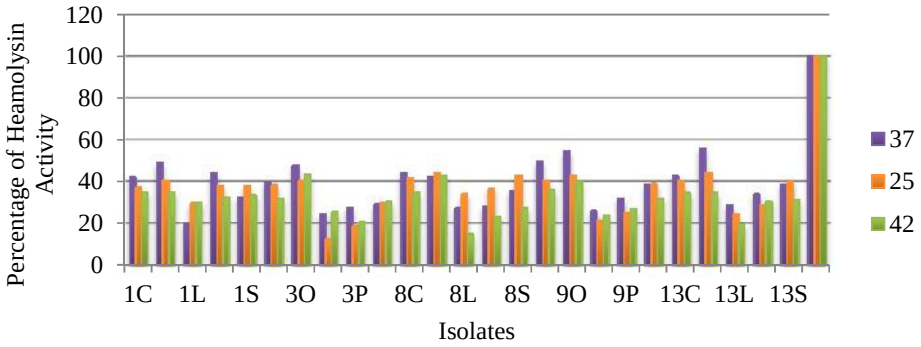


FIGURE 5: Percentage of Heamolysin activity at (0.01%) of Fatty Acids at Different Temperatures

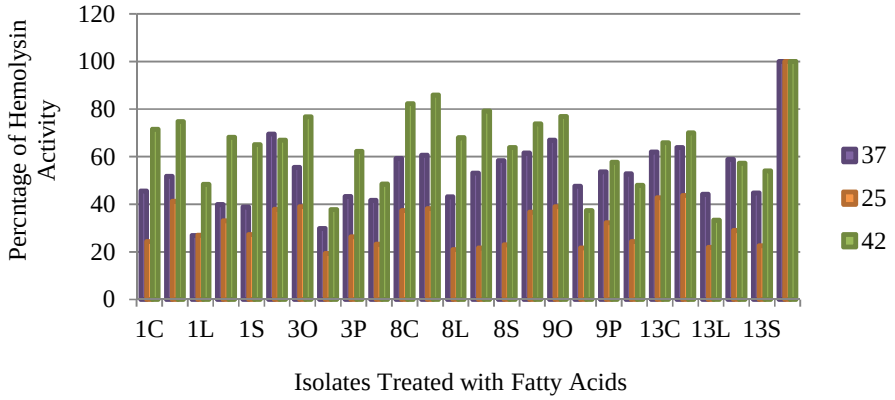


FIGURE 6: Percentage of Heamolysin Activity at (0.005%) of Fatty Acids at Different Temperatures

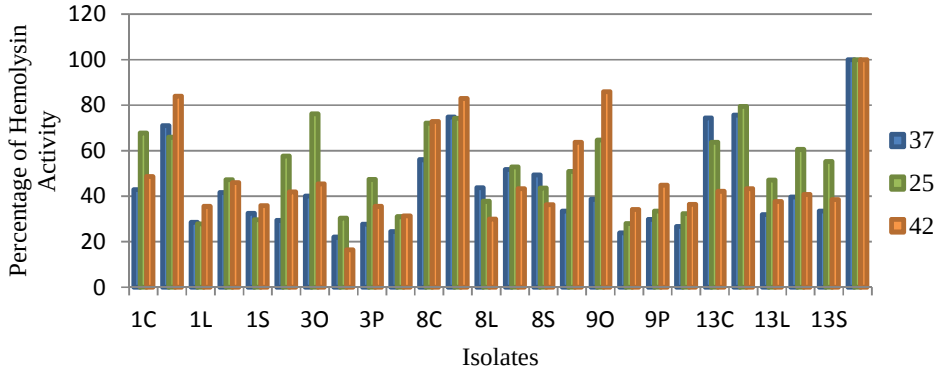


FIGURE 7: Percentage of Heamolysin Activity at (0.0025%) of Fatty Acids at Different Temperatures

These results were agreed with the study of [33] because the percentages have decreased from (52.9%) to (5.9%), she reported that all acid concentrations have been inhibited heamolysin production or interfere with its action, that may be attributed to ability of lauric acid to inhibit signal transport at cytoplasmic membrane leading to modification membrane structure then morphological structure of carrier signal proteins cross membrane (34).While(8) have been referred to effect of fatty acids onrsbA gene that organize swarming &heamolysin production in *P. mirabilis*, the srains that have the gene were influenced but those which lost the gene didn't influenced.

Effect of Fatty Acids on Biofilm Formation

Biofilms produced by *P. mirabilis* causes very dangerous problems for catheterized patients so inhibition of biofilm formation is the most important aim of this study. Detection of biofilm production by bacterial isolates in recent study have been done by cultivation the tested isolates (1,3,8,9,13) on LB agar treated & untreated with the fatty acids concentrations then bacterial suspension have been transported to 96 well microtiter plates. O.D were determined by ELISA at 630 nanometer to estimate the quantity of produced biofilms that adhered on the surface of well. Results have been showed that all tested isolates were able to produce biofilms, the tables2,3,4 demonstrate O.D values with & without three concentrations of four fatty acids:

TABLE 2: Effect of Fatty Acids (0.01%) on Biofilm Formation

Fatty Acids / Isolates	C	OD			
		L	O	P	S
1	0.223	0.040	0.042	0.086	0.088
3	0.218	0.039	0.050	0.093	0.088
8	0.222	0.037	**0.038**	0.085	0.092
9	0.225	**0.034**	0.041	0.064	0.081
13	0.223	0.038	0.039	0.099	0.088

L= Lauric acid, P= Palmitic acid, O=Oleic acid, S= Stearic acid, C=Control

TABLE 3: Effect of Fatty Acids (0.005%) on Biofilm Formation

Fatty Acids / Isolates	C	OD			
		L	O	P	S
1	0.223	0.040	0.077	0.097	0.082
3	0.218	0.046	0.062	0.143	0.107
8	0.222	**0.039**	0.049	0.117	0.100
9	0.225	0.042	**0.043**	0.061	0.073
13	0.223	0.043	0.047	0.092	0.083

L= Lauric acid, P= Palmitic acid, O=Oleic acid, S= Stearic acid, C=Control

TABLE 4: Effect of Fatty Acids (0.0025%) on Biofilm Formation

Fatty Acids / Isolates	C	OD			
		L	O	P	S
1	0.223	0.037	0.077	0.207	0.205
3	0.218	0.055	0.111	0.201	0.208
8	0.222	0.048	0.090	0.218	0.203
9	0.225	0.037	0.065	**0.219**	**0.221**
13	0.223	**0.036**	0.082	0.208	0.212

L= Lauric acid, P= Palmitic acid, O=Oleic acid, S= Stearic acid, C=Control

Clear effect was seen of fatty acids specially for lauric acid & oleic acid at (0.01%, 0.005%), inhibition was increased as concentration increased, statistical analysis have showed considerable significant differences at the degree ($P \geq 0.05$) between optical density of tested isolates & with control group. Adding of long chain fatty acids prevent adhesion of S*taphylococcus aureus* on non-living surfaces and delaybiofilm formation[35], another study documented that saturated fatty acids inhibit biofilm formation of S. *aureus* & *P. aeruginosa* at minimum inhibitory concentration (MIC), fatty acids were bactericidal but the mechanism was not known, it may be disrupt & degrade the cell wall resulting in biofilm formation inhibition[36].

Effect of Fatty Acids on Sugar Content of LPS

Treatment of sugar extract of LPS with fatty acids have an inhibitory effect, most effective acid was lauric acid that the sugar content decreased after exposure to (0.01%) of this acid, in contrast to oleic acid which increased the sugar content in LPS as shown in table (5):

TABLE 5: Effect of Fatty Acids on Sugar Content of LPS

Isolates		Acid Conc.	
		0.01%	0.005%
		(S) µg/ml	
1	C	100.1	100.1
	O	22.3	201.2
	L	11.1	95.3
	P	11.3	100
	S	16.9	97.4
3	C	88.6	88.6
	O	30.3	107.2
	L	11.6	59.6
	P	20.7	78.1
	S	23.7	86
8	C	95.7	95.7
	O	30.7	200.4
	L	11.7	18.2
	P	23.7	81.6
	S	25.5	85.1
9	C	103.1	103.1
	O	40.6	201.5
	L	22.1	67.9
	P	28.5	100.7
	S	33	102.9
13	C	188.1	188.1
	O	134.4	225.4
	L	40.1	176.9
	P	44.9	170.8
	S	105.4	185.2

C= Control, O= Oleic acid, P= Palmitic acid, L=Lauric acid, S= Stearic acid, F=Fat in LPS

TABLE 6: Effetct of Fatty Acids on Fat Content of LPS

Isolates		Acid Conc.	
		0.01%	0.005%
		(F) µg/ml	
1	C	63	63
	O	36	61
	L	**2**	**41**
	P	15	55
	S	25	57.5
3	C	48	48
	O	40	46.5
	L	**6**	**18**
	P	26	40
	S	13	47.5
8	C	55	55
	O	29	53.5
	L	**15**	46
	P	16	50
	S	26	54
9	C	60	60
	O	37	57.5
	L	**20**	40
	P	31	59
	S	32	59.5
13	C	50	50
	O	23	50
	L	**6**	**16**
	P	**8**	48
	S	12	34

C= Control, O= Oleic acid, P= Palmitic acid, L=Lauric acid, S= Stearic acid, F=Fat in LPS

Because there are no significant differences appeared between the results of (0.005%) & (0.0025%) so the results of the last concentration have been neglected. Statistical analysis has showed significant differences between the effects of different acids in the sugar content. LPS is important virulent factor so any decrease of the sugar content will change O-side chain, and loss of virulence if the side chain is lost or modified[37].

Effect of Fatty Acids on Lipid Content

Lipid -A- is the inner part of LPS and it represents the endotoxin of gram negative pathogens, the pyrogenic factor in infectious diseases. The following equation has been applied to determinate the lipid content of LPS:

$$Total\ Lipd\ (\mu g/ml) = \frac{sample\ absorption\ spectrum}{standard\ solution\ absorption\ spectrum} \times Standard\ Solution\ Conc.$$

The calculated shown in the table below were obtained after treatment with the tested fatty acids (0.01% & 0.005%) conc.:

Positive effect of fatty acids especially for lauric was noticed in decreasing the lipid content of LPS, leading to inhibition of bacterial pathogenecity. The amounts of lipid were agreed with[38] who extracted LPS from Brucella *spp.* The means were (91.9 & 82.1) µg /ml but disagreed with the results of [39] which reported the amount of lipid extracted from LPS of *Klebsiella oxytoca* was (266) µg/ml. We concluded from our results the ability of some fatty acids of inhibition the virulence factors of *P. mirabilis* that isolated from catheterized patients.

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