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Cihan University-Erbil Scientific Journal (CUESJ)

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Research Article

Effect of Drinking Water Hardness on Kidney Stones Formation in Ranya District

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ABSTRACT

This study was conducted in Rania District, Raparin University, during September 2018–March 2019, to test the relation between water hardness and kidney stone formation. The investigation depended on questionnaire form which was distributed on 100 person in Raparin (Rania, Hajiawa, and Chwarqurna) and patients whom vested the Rania clinical during December 1, 2018–January 22, 2019 which were 238 patients and only 20 of them had kidney stones developing which represent 8.4% of the total kidney diseases. The results indicated to significant effect of gender at level of significant 5% on kidney stones formation, 10% of male, and 18% of female having kidney stones. The results of Chi-square test indicated to highly significant effect of age on kidney stone formation at level of significant (0.001). The kidney stone formation increased from 19.23% to 75% with an increase in age class from (14–34) to (54 or more) year. The negative correlation coefficient value of ($r = -0.63^*$) was recorded between water hardness and stone risk index due to the high magnesium content of drinking water in the studied area.

Keywords: Ca-hardness, component, Mg-hardness, stone risk index, total water hardness

INTRODUCTION

The urolithiasis regards as a very complex disease, many factors affecting on urinary calculus or formation of kidney stones.[1]

Hard water means the water which contains more minerals in comparing with ordinary water. The most important ions are calcium and magnesium. Hardness is expressed in terms of calcium carbonate ($CaCO_3$) mg/l. The degree of hardness increases with increasing the concentration of calcium and magnesium in water if the concentration of them <75 m/l is considered as a soft water, 76–150 mg/l moderately hard, and more than 150 mg/l regards as a hard water.[2]

Numerous studies were conducted in the Kurdistan region about water quality and its suitability for drinking purpose, most of them included total hardness, magnesium hardness, and calcium hardness water which directly related to kidney stones developing or forming or regards as one of the factors related to kidney stone formation in the world depending on type of water hardness and magnesium and bicarbonate concentration in drinking water.[1,3]

Several factors increase the risk for kidney stones forming or developing which were summarized as inadequate water or fluid intake and dehydration, reduce in urinary volume, and increase in concentrations or levels of certain chemicals in urine such as calcium, oxalate, and uric acid to high level or decrease of magnesium to low level or too low and some medical conditions such as reflux and medullary sponge kidney urinary tract infections in additional to tubular acidosis. Anything that reduces or blocks the urine flow like urinary obstruction and genetic abnormalities also increase the risk of kidney stones developing.[4]

Drinking water hardness in the Kurdistan region studied by numerous researchers as follow:

The water harness in Sulaimani governorate the results indicated that the total hardness was ranged (from 110 to 280) mg $CaCO_3$/l, it means most of the studied water hard and may have negative effect of kidney stone developing.[5]

The water harness study of (Chaq–Chaq) Kliassan stream in Sulaimani city showed that the total hardness was ranged from 110 to 355 mg/l; it means most of studied water hard

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to very hard and could have negative effect of kidney stone formation.[6]

The water hardness of karees, springs, and streams in Erbil governorate was studied by Esmail,[7] the results explained that the total hardness was ranged from 151 to more than 1000 mg/l with a mean of 321 mg/l. It means most of the studied water was hard to very hard.

The water hardness of Kapran near Erbil city was studied, the results indicated that the total hardness was ranged between 135.48 and 306.93 mg means must studied moderate hard to very hard.[8]

The water hardness of springs at Halgwrd mountain was ranged between 93 and 122 mg/l $CaCO_3$, it means that the water of this location is must soft water and suitable for drinking purpose depending on.[9,10]

The water hardness in Erbil was ranged (138–446) mg $CaCO_3$/l, it means most of the studied water was hard to very hard.[11]

The value of total hardness of the water in Bator and Southwest and south Erbil was ranged between 173 and 900 mg $CaCO_3$/l, this indicated that most studied water had hard to very hard class or not suitable for drinking purpose[9] or may have negative effect kidney stone developing.[8,12]

The mean of total hardness, Ca-hardness, and Mg-hardness was (248.82–339.30), (207.49–291.21), and (41.34–97.57) mg/l, respectively, for drinking water in Halabja as mentioned by Schwart *et al*.[13]

The studied water hardness in Dohuk governorate had the values ranged between 281 and 972 mg/l as $CaCO_3$; it means most of the studied water had hard to very hard class.[14]

The water hardness of the studied water in Sulaimani governorate recorded the total hardness of (200–580) mg/l as $CaCO_3$ means; it refers to that the studied water has hard to very hard class and may have negative effect on kidney stone forming.[15]

The inverse relationship was recorded between drinking water hardness and kidney stones developing (urolithiasis) for 2302 patients in various geographical regions of the united state.[16]

The workers found the positive correlation between drinking water hardness and kidney stones forming and developing (urolithiasis) in 1000 general hospitals in the United States of America. On the other hand, the weak correlation was recorded between kidney stone-forming and drinking water hardness as mentioned by Churchill *et al*.[17] and Shuster *et al*.[18]

The positive correlation was recorded between total water hardness and kidney stones forming in the United Kingdom, due to the water hardness values in South and East of England.[19]

The relation between drinking water quality (hardness) and the stone risk index (SRI) (kidney stone forming) for 24 Provincial Capitals of Iran, was studied by Basiri *et al*.[1] The results indicated that the water hardness was ranged between 57 and 874 PPM; the SRI was ranged between 0.00 and 0.0198. The non-significant correlation was recorded between total water hardness of tap water and kidney stone formation, while the negative significant correlation was recorded between Mg-hardness and kidney stone formation with the correlation coefficient value of $r = -0.51$*.

In the study conducted in Iran from 20 capital cites depending on large number of samples (21200) the negative significant correlation means increase in water hardness cased decrease in risk of kidney stone forming since 30% of total hardness is magnesium hardness which causes decrease in stone formation.

Since the drinking water of the Kurdistan region is hard, especially the water of springs, wells, and rivers in Raparin District is hard to very hard which ranged from 150.46 to 1414.80 with the mean value of 337.71 mg/l as $CaCO_3$. It means the water hardness of water in the Kurdistan region is above the recommended standard value of WHO.[9]

For the above reasons, the aims of this investigation are to study the effect of:

1. Drinking water hardness on kidney stones developing or formation.
2. Amount of water intake per day, gender, age, and family history on developing kidney stones.

MATERIALS AND METHODS

For scientific research about the effect of drinking water hardness on kidney stones forming, the data were collected from December 1, 2018 to January 20, 2019 in one clinic of Rania city specialized in kidney diseases. The number of patients visited the mentioned clinic was 238 patients and only 20 of them had kidney stones developing or forming.

The other source of data collecting was the distribution of questionnaire form on 100 persons (50 male and 50 female) inside and outside of Raparin University, which included (Hajyawa, Chwarqwrna, and Ranya), the questionnaire form was shown from Table 1 which prepared as mentioned from 20.

The survey was conduct on water hardness in the Kurdistan region, while 52 water samples in Raparin District was depended in calculating risk index of stone formation. The water sample was analyzed from Raparin during 2018 by Dawson,[20] the total water hardness, magnesium hardness, and calcium hardness we determined according to following models and stone risk formation for 52 samples of water were calculate using following equation as mentioned by Basiri *et al*.[1]

Stone risk index = SRI = [(Ca mg/l)/(Mg mg/l)]/(bicarbonate (mg/l).

Table 1: The questionnaire form

Age	**Gender**		
	Male	**Female**	
Source of drinking water	Spring	Well	
Amount of red meat eat	High	Medium	Low
Amount of water intake L/day	3–4	2–3	1–2 L/day
Family history	Yes	No	

Total hardness = [Ca mg/l*2.50+Mg mg/l*4.20].
Mg-hardness = (Mg mg/l*4.20).
Ca-hardness = Total hardness−Mg-hardness.

The statistical analysis was conduct using Chi-square test depend on the SPSS program version (22) and regression correlation curve was drown using Excel program.

RESULTS

As shown from Table 2, the gender affected significantly on kidney stones forming at level of significance (0.05), the statistical analysis indicated that the calculated Chi-square value (3.81) is more than tabulated value (3.50). The results indicated that 10% of male and 18% of female having kidney stones forming depending on total sample size (100). On the other hand, if the percentage was calculated separately for male and female, the percentage of samples having kidney stones forming was 20 and 30% for male and female, respectively.

Table 2: The effect of gender on kidney stone

Gender	Kidney stone		Percentage of patients having kidney	
	Have	Have not	From subsample	From total (*n*=100)
Male	10	40	20	10
Female	18	32	30	18
Total	28	72		28
Calculated Chi-square=3.81		Tab. Chi-square=3.50		*P*≥0.05

Table 3 indicates to the highly significant effect of age on kidney stone formation at the level of significant (0.001) with the calculated Chi-square value of 15.50. The kidney stone formation increased from 19.23% to 75% with increase in age.

Table 4 indicates to the effect of source of drinking water on kidney stone was not significant in spite of the percentage of kidney stone in case of a source of drinking spring water was 20%, while the percentage was 29.415 in case of drinking well water.

Table 5 explains that the amount of consumed meat per person had not affected significantly on kidney stones. Chi-square test refers to non-significant effect since the calculated Chi-square value (1.29) is less than tabulated value (3.40).

Table 6 explains that the amount of drinking water (L/day/person) had not affected significantly on kidney stone formation. Chi-square test refers to non-significant effect since the calculated Chi-square value (1.290) is less than table value.

Table 3: Explains the effect of age on kidney stone

Age (year)	Have kidney stone	Have not kidney stone	Percentage of patients having kidney stone
14–34	16	67	19.23
34–54	9	4	69.23
54 or more	3	1	75.00
Calculated Chi-square value (X^2)=15.50***		Tab. Chi-square=3.20	*P*≥0.001

Table 4: Indicates to the effect of source of drinking water on kidney stone

Source of water	Have kidney stone	Have not kidney stone	Percentage of patients having kidney stone
Spring	3	12	20.00
Well	25	60	29.41
Calculated Chi-square=3.00		Tab. Chi-square=3.50	*P*≥0.15

Table 5: Indicates to the effect of eating meat on kidney stone

Meat consumption	Have kidney	Have not kidney stone	% of patients having kidney stone
High	7	25	21.88
Medium	12	23	34.29
Low	9	24	27.27
Calculated Chi-square=1.29		Tab. Chi-square=3.40	*P*≥0.53

Table 6: Indicates to the effect amount of drunken water (L/day) on kidney stone-forming

Amount of drunk water L/day	Have kidney stone	Have not kidney stone	Percentage of patients having kidney stone
Low (1–2)	16	33	32.65
Medium (2–3)	6	15	28.57
High (3–4)	6	24	20.00
Calculated Chi-square=1.47		Tab. value=3.20	*P*≥0.47

Table 7: Effect of family history on kidney stone formation

Family history	Have kidney stone	Have not kidney stone	Percentage of patients having kidney stone
Yes	11	23	32.35
No	17	49	25.76
Calculated Chi-square=0.48		Tab. value=3.50	$P \geq 0.49$

Table 8: Explains some important chemical properties of water samples in Raparin District

Location	Concentration (mg/L)			SRI	Hardness (mg/$CaCO_3$/L)		
	Mg^{2+}	Ca^{2+}	HCO_3		Mg^{2+}	Ca^{2+}	HCO_3
1-Spring	17.92	61.80	301.65	0.42	73.63	153.88	227.52
2-River	10.27	44.18	286.88	0.55	42.22	110.01	152.23
3-Spring	56.93	267.50	3057.93	0.06	233.97	666.08	900.05
4-Well	30.49	128.20	1653.10	0.09	125.32	319.22	444.54
5-Well	23.64	102.80	547.48	0.29	97.16	255.97	353.13
6-Well	23.02	91.86	474.46	0.31	94.60	228.73	323.33
7-Spring	19.93	59.78	318.54	0.34	81.92	148.85	230.77
8-Well	17.53	44.62	256.75	0.36	72.06	111.10	183.16
9-River	31.42	65.08	1164.49	0.07	129.12	162.05	291.17
10-Spring	11.94	50.48	238.39	0.65	49.07	125.70	174.77
11-Spring	17.11	88.30	454.08	0.42	70.33	219.87	290.20
12-Spring	20.81	88.52	402.05	0.39	85.52	220.41	305.94
13-Well	12.55	209.80	990.03	0.62	51.59	522.40	573.99
14-Well	15.72	146.00	735.66	0.46	64.61	363.54	428.15
15-Well	19.68	69.40	418.22	0.31	80.88	172.81	253.69
16-Well	11.81	63.16	273.65	0.72	48.53	157.27	205.80
17-Well	21.60	96.34	506.91	0.32	88.78	239.89	328.66
18-Well	24.24	83.22	408.64	0.31	99.63	207.22	306.84
19-Well	30.36	100.80	513.80	0.24	124.78	250.99	375.77
20-Spring	28.30	89.88	433.77	0.27	116.30	223.80	340.10
21-Spring	26.56	78.10	387.17	0.28	109.15	194.47	303.61
22-Spring	94.99	411.40	1808.65	0.09	390.42	1024.39	1414.80
23-Spring	26.74	65.44	349.90	0.26	109.88	162.95	272.83
24-Spring	28.30	78.90	409.86	0.25	116.30	196.46	312.76
25-Spring	26.44	69.08	356.67	0.27	108.65	172.01	280.66
26-Spring	26.71	70.06	371.55	0.26	109.79	174.45	284.24
27-Spring	12.22	40.26	193.68	0.62	50.21	100.25	150.46
28-Spring	63.29	234.80	1091.90	0.12	260.11	584.65	844.77
29-Spring	38.04	108.90	575.29	0.18	156.34	271.16	427.51
30-Well	12.88	55.38	257.73	0.61	52.92	137.90	190.82
31-Well	15.78	60.48	296.95	0.47	64.86	150.60	215.45
32-Well	14.42	55.46	271.51	0.52	59.28	138.10	197.38
33-Spring	29.57	95.16	459.33	0.26	121.52	236.95	358.47
34-Spring	31.03	113.70	527.47	0.25	127.54	283.11	410.65
35-River	41.88	99.14	551.32	0.16	172.13	246.86	418.99
36-Spring	20.29	64.86	315.92	0.37	83.40	161.50	244.90
37-Spring	13.08	43.92	220.33	0.56	53.76	109.36	163.12

(*contd...*)

Table 8: *(Continued)*

Location	Concentration (mg/L)			SRI	Hardness (mg/$CaCO_3$/L)		
	Mg^{2+}	Ca^{2+}	HCO_3		Mg^{2+}	Ca^{2+}	HCO_3
38-Spring	18.40	69.06	328.12	0.42	75.61	171.96	247.57
39-Well	10.76	61.48	270.35	0.77	44.24	153.09	197.33
40-Spring	17.15	53.76	265.11	0.43	70.48	133.86	204.34
41-Well	18.06	77.78	355.94	0.44	74.23	193.67	267.90
42-Well	19.45	60.42	304.45	0.37	79.95	150.45	230.39
43-Well	23.62	80.14	388.39	0.32	97.06	199.55	296.61
44-Well	21.78	76.40	406.50	0.32	89.52	190.24	279.75
45-Well	27.19	91.88	451.03	0.27	111.76	228.78	340.54
46-Well	34.02	69.10	412.85	0.18	139.82	172.06	311.88
47-Spring	17.22	60.98	297.50	0.44	70.77	151.84	222.61
48-Well	24.38	84.04	416.33	0.30	100.22	209.26	309.48
49-Well	41.02	84.52	553.15	0.14	168.58	210.45	379.03
50-Well	25.16	101.50	476.59	0.31	103.42	252.74	356.16
51-Well	24.96	92.58	442.01	0.31	102.59	230.52	333.11
52-Spring	26.51	118.10	613.05	0.27	108.95	294.07	403.02
Min	10.27	40.26	193.68	0.06	42.22	100.25	150.46
Max	94.99	411.40	3057.93	0.77	390.42	1024.39	1414.80
Average	25.33	93.82	535.83	0.35	104.11	233.61	337.71

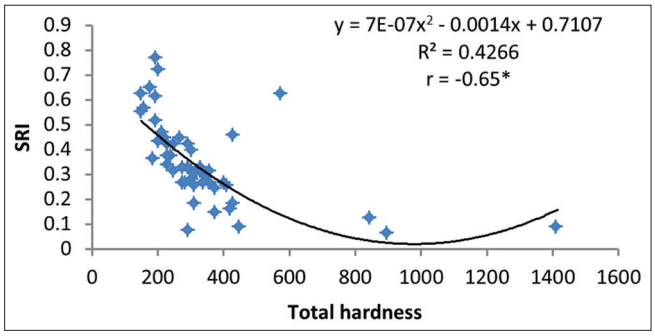

Figure 1: Relation between total hardness of drinking water and stone risk index of kidney

Table 7 explains that the family history of the studied samples person had not affected significantly on kidney stone formation. Chi-square test refers to non-significant effect since the calculated Chi-square value (0.484) is less than tabulated value.

Figure 1 shows the correlation between SRI and total hardness of 52 water resources (rivers wells and springs) in Raparin District. The coefficient of determination (R^2) and correlation coefficient (r) between them was 0.426 and −0.65*, respectively. The SRI, coefficient of determination (R^2), and correlation coefficient (r) were determined from the data recorded in Table 8.

DISCUSSION

Table 2 shows that the gender affected significantly on kidney stone formation the highest value (18%) was recorded from female, while (10%) was recorded from male this may be due to human activity of male more than female or may be due to biological difference male and female and lifestyle.

Table 3 refers to highly significant effect of age on kidney stone (0.001). The kidney stone information increased from 19.25% to 75% for age class of (14–34) year to age class of more than (54) year, respectively, since every 10 years the kidney stone formation is possible as mention by Basiri *et al.*[1] and Kumar *et al.*[4] The statistical analysis using Chi-square test also indicated to significant effect of age on kidney stone formation since the calculated Chi-square value (15.50) was more than tabulated value which was equal to 3.50. On the other hand, the reverse results can be obtained depending on total sample size of this study because the sample size increased with decrease in age.

Table 4 explains that non-significant effect of source of drinking water on kidney stone formation or there was no significant difference between water hardness of spring water and well water since both of them are hard water to very hard water.[9]

The Mg-hardness represents 31% of total hardness as shown from Table 8, similar results were obtained by Basiri *et al.*[1] in the main cities of Iran which caused decrease in risk of stone formation. The significant negative correlation coefficient was recorded between the risk of stone formation and total hardness of 52 water samples in Raparin District with the value of $r = -0.65^{**}$ as shown from Figure 1 which decrease in the effect of total hardness of drinking water in kidney stone formation. These results agree with those recorded by Basiri *et al.*[1]

Table 5 explains that consumed meat not affected significantly on kidney stone formation in spite of the

difference in the percentage of kidney stone formation which ranged between (21.88% and 34.29%).

Table 6 indicates to decrease in kidney stone formation from 32.65% to 20% with increase amount drunk water per day or water intake per day per person, this may be due to increase water may be prevent precipitation of crystals such as calcium, oxalate, and uric acid in the kidney then decrease stone formation.

As shown from Table 7, the family history caused an increase in percentage of kidney stone formation from 25.76% to 32.35% but not reached to the significant effect.

Its appear from the above discussion that there is more than one factor responsible for kidney stone formation for this reason, sometime the result for single factor is not significant, while the combination among the studied factors may cause the kidney stone formation.

CONCLUSION AND RECOMMENDATIONS

The kidney stone formation affected significantly by gender and amount of drunken water (L/day/person). The total water hardness was not affected significantly on kidney stone formation since magnesium hardness represents 31% of total hardness which caused decrease in the risk of kidney stone formation. The drinking water in Raparin District is hard to very hard, but they have not risk on kidney formation due to high magnesium and bicarbonate of the studied water resources.

We highly recommend that:

- It is necessary to increase sample size.
- It is better to have the same number of studied samples for each age classes.
- It is better to have the same number of male and female number in the suture studies.

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Research Article

Characterization of *Lactobacillus* Isolates from Human Mouth and Feces as Probiotics

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ABSTRACT

Probiotics are live microbes that give many health benefits to human beings and animals, the most studied and commonly used probiotics are Gram-positive bacteria; lactobacilli and bifidobacteria. At nowadays, *Lactobacillus* spp. constitute more than two-thirds of the total numbers of probiotic species. The present study aimed to characterize *Lactobacillus* that locally isolated from human mouth and feces as probiotics. A total of three *Lactobacillus* isolates; *Lactobacillus fermentum* Lb2, *Lactobacillus rhamnosus* Lb9, and *Lactobacillus paracasei* Lb10 were investigated in respect to acid and bile salts tolerance, antibiotics susceptibility, and cell surface hydrophobicity *in vitro* using bacterial adhesion to hydrocarbons method. In comparison with the other two isolates, the isolate *L. fermentum* Lb2 was able to grow in all pH values and in the presence of different concentrations of bile salts. Antibiotics susceptibility profile showed that the tested *Lactobacillus* isolates were sensitive to ampicillin, amoxicillin, and erythromycin, while they were resistant to the other antibiotics that used in this study. *L. fermentum* Lb2 exhibited high surface hydrophobicity (77.26%), while the other tested isolates; *L. rhamnosus* Lb9 and *L. paracasei* Lb10 revealed moderate adhesion abilities, 68.56% and 65%, respectively. *L. fermentum* Lb2 exhibited good probiotic behavior with respect to acid and bile salt tolerance as well as adhesion ability to hydrocarbons.

Keywords: Acid and bile salt tolerance, cell surface hydrophobicity, *Lactobacillus*, probiotic

INTRODUCTION

The attention of probiotic as an effective approach to prevent and treat a wide range of diseases is observed in recent years.[1] The term probiotic consists of two words which are pro, means for, and biotic, means life and totally means for life.[2] In 2001, the Food and Agriculture Organization of the United Nations and World Health Organization defined probiotics as "Live microorganisms which, when administered in adequate amounts, confer a health benefit on the host."[3] The US Food and Drug Administration stated that the probiotic is considered as generally recognized as safe.[4] Many studies showed that probiotics are used effectively to do many beneficial functions to the host[5-9] when they are taken in adequate quantity, and the recommended dosage of them is ranged usually between 10^8 and 10^{10} CFU/day.[10]

Probiotics can be found in a wide assortment of commercial dairy products[11] as well as they can be prepared as capsules, powder, granules, and fermented or pelleted feed.[12] Microorganisms are considered as probiotic when they have several criteria such as the ability to adhesion to gastrointestinal tract, the ability to tolerance acidity and bile salts, and the resistance to antibiotics[13] have health benefits for the host[14] and safety.[15] The most commonly used probiotics are *Lactobacillus* and *Bifidobacterium* species.[16] Lactobacilli are the largest group included in lactic acid bacteria[15] and have many functions, such as improvement of growth performance and health of gastrointestinal tract, this answers why they are among the bacteria mostly used as probiotics in animal feeds and human foods.[17,18] The aim of this study was to characterize three locally *Lactobacillus* isolates as probiotics.

MATERIALS AND METHODS

Bacterial Isolates

A total of three locally *Lactobacillus* isolates were used in this study; *Lactobacillus fermentum* Lb2 that isolated from the mouth, *Lactobacillus rhamnosus* Lb9 and *Lactobacillus paracasei* Lb10 that isolated from feces.[19]

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Acid Tolerance Test

Fresh broth cultures of *Lactobacillus* isolates (Lb2, Lb9, and Lb10) were used at concentration of 1.5×10^8 CFU/ml according to McFarland tube No. 0.5 (Hardy Diagnostics, Santa Maria). Serial dilutions were made in tubes contain MRS broth (Oxoid, Germany), then bacteria were transferred individually to MRS broth at various pH values (2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, and 6), and tubes were incubated at different times (1, 1.5, 2, 2.5, and 3 h) h; thereafter, 0.1 ml from each culture was taken and spread on MRS agar (Oxoid, Germany) with two replicates. Plates were incubated anaerobically for 24 h at 35°C. The pronounced colonies were counted, and results were recorded corresponding to control.[20]

Bile Salt Tolerance Test

Fresh broth cultures of *Lactobacillus* isolates (Lb2, Lb9, and Lb10) were used at the concentration of 1.5×10^8 CFU/ml according to McFarland tube No. 0.5. Serial dilutions were made in tubes contain MRS broth, then bacteria were transferred individually to MRS broth supplemented with various concentrations of bile salts (Difco, USA) (0.3%, 0.5%, and 0.7%), tubes were incubated at different times (1, 1.5, 2, 2.5, and 3 h); thereafter, 0.1 ml from each culture was taken and spread on MRS agar with two replicates, and then plates were incubated anaerobically for 24 h at 35°C. The pronounced colonies were counted, and results were recorded corresponding to control.[21] The optical density of bacterial isolates growth in each bile salt concentration was measured at wavelength 660 nm after 3 h of incubation.

Antibiotics Susceptibility Test

Fresh broth cultures of *Lactobacillus* isolates (Lb2, Lb9, and Lb10) were used at concentration of 1.5×10^8 CFU/ml according to McFarland tube No 0.5; thereafter, 0.1 ml from each culture was taken and spread on MRS agar, and then antibiotics discs (25 μg ampicillin, 25 μg amoxicillin, 10 μg tetracycline, 1.25/23.75 μg trimethoprim-sulfamethoxazole, 10 μg erythromycin, 10 μg gentamycin, 25 μg streptomycin, 10 μg amikacin, 10 μg neomycin, and 10 μg chloramphenicol) (Bioanalyse, Turkey) were gently placed on MRS agar plates, plates were incubated anaerobically at 35°C for 24 h. The inhibition zones were measured and recorded; the work was done with two replicates.[22]

Cell Surface Hydrophobicity Test

It was determined *in vitro* using bacterial adhesion to hydrocarbons (BATH) method,[23] a fresh cultures of *Lactobacillus* isolates were subjected to centrifugation (6000 rpm, 10 min), cells were washed by phosphate buffer saline (Oxoid, Germany), resuspended in the same buffer and the optical densities of suspensions were adjusted to 0.8–1 at (OD 560), and the data were recorded as A 0.6 ml of *Lactobacillus* suspensions were mixed with 1.2 ml of n-hexadecane (Sigma, USA), gently vortexed, and tubes were incubated anaerobically at 35°C for 30min. The mixtures were vortexed rigorously for 2 min and then allowed to stand for 15 min at room temperature to ensure complete separation of the organic and the aqueous phase. The absorbance of aqueous phase was measured at 560 nm and recorded as A, the affinity to solvent was expressed using the formula:

$$\text{Hydrophobicity percentage} = \frac{(\text{A0-A})}{\text{A0}} \times 100$$

A0, before adding n-hexadecane; A, after adding n-hexadecane.

Statistical Analysis

Statistical Analysis System[24] program was used to show the effect of different factors in study parameters. Furthermore, least significant difference (LSD) test was used to significant compare between means in this study.

RESULTS AND DISCUSSION

Probiotic strains must be able to survive and colonize in the presence of acidic conditions and bile salt so as to travel through the gastrointestinal tract and reach to digestive tract where they give their therapeutic effect to host.[25] In current study, three *Lactobacillus* isolates were subjected to different pH values with different incubation periods to examine their ability to tolerate acidic conditions. According to the results in Table 1, all three isolates were unable to grow at pH 2, while they were able to grow at all times of incubation at pH 4, 4.5, and 5 with a decline in their growth at pH 6 after its rise at pH 5.5. At pH 5, there was no significant difference in the growth of the three isolates except the 1st h of incubation with significant difference in time 3 h for Lb9.

The gradual decrease in the survival rate of bacterial growth, from pH 5 down to pH 2, as well as the decrease in bacterial growth at pH 6 after its increasing at pH 5.5 can be observed, it seemed there is an affinity of the three isolates to pH 5.5 where their survival percentage reached the highest at this pH value, these results are similar to previous studies that used pH 5.5 in preparation of media as it allows optimum growth for *Lactobacillus*.[26,27] At pH 6, the reduction of the growth of the three isolates is noticeable at the 1st h of incubation while there was no significant difference in their growth in times 1.5 and 2 h for Lb2 and in times 2 and 2.5 h for Lb10. The significant decrease in the bacterial growth at the 1st h of incubation is due to the sudden change in pH as reported by Fernández-Calviño and Bååth.[28]

The ability of three *Lactobacillus* isolates to survive at three different concentrations of bile salts for different incubation periods was examined. The results in Table 2 show that all isolates were able to survive at all bile salts concentrations. The results of this study are agreed with previous studies used 0.3% and 0.5% of bile salts concentrations for 2.5 h of incubation.[29,30] The gradual decrease in the survival rate of bacterial growth as a result of the increase of concentration of bile salts can be observed. The significant decrease in bacterial growth at the 1st h of incubation is due to the sudden exposure to bile salts stress while there was no significant decrease in bacterial growth after the 1st h of incubation particularly at high concentration of bile salts. The resistance of *Lactobacillus* to bile salts may be due to different mechanisms such as changes in the composition of cell membrane and cell wall, active efflux of bile acids, and bile salt hydrolysis.[31] Figure 1 shows

Table 1: Acid tolerance percentages of *Lactobacillus* isolates at different pH values

pH	h	Mean of viable count (log10 CFU/ml)			Survival percentage		
		Lb2	Lb9	Lb10	Lb2	Lb9	Lb10
2.5	1	8.505^{B}	8.612^{B}	-	2	3	0
3	1	8.748^{B}	8.959^{B}	8.643^{B}			
	1.5	-	8.913^{a}	-	4	25	4
	2	-	8.838^{a}	-			
	2.5	-	8.643^{b}	-			
3.5	1	8.851^{B}	9.017^{B}	8.799^{B}			
	1.5	8.740^{a}	8.949^{a}	8.505^{b}			
	2	-	8.886^{b}	-	10	33	9
	2.5	-	8.770^{b}	-			
	3	-	8.643^{b}	-			
4	1	9.120^{B}	9.086^{B}	9.004^{B}			
	1.5	9.110^{a}	9.079^{a}	8.959^{a}			
	2	9.008^{b}	9.004^{a}	8.869^{a}	46	45	39
	2.5	8.954^{b}	8.949^{b}	8.819^{b}			
	3	8.919^{b}	8.892^{b}	8.698^{b}			
4.5	1	9.178^{B}	9.264^{B}	9.093^{B}			
	1.5	9.152^{a}	9.136^{b}	9.096^{a}			
	2	9.123^{a}	9.113^{b}	9.079^{a}	55	59	61
	2.5	9.079^{b}	9.071^{b}	9.060^{a}			
	3	8.963^{b}	9.004^{b}	9.045^{a}			
5	1	9.274^{B}	9.204^{B}	9.152^{B}			
	1.5	9.276^{a}	9.198^{a}	9.130^{a}			
	2	9.247^{a}	9.146^{a}	9.113^{a}	77	64	66
	2.5	9.235^{a}	9.130^{a}	9.086^{a}			
	3	9.227^{a}	9.123^{b}	9.082^{a}			
5.5	1	9.298^{B}	9.301^{A}	9.250^{A}			
	1.5	9.294^{a}	9.278^{a}	9.230^{a}			
	2	9.271^{a}	9.247^{a}	9.204^{a}	81	80	80
	2.5	9.255^{a}	9.230^{b}	9.158^{b}			
	3	9.247^{a}	9.225^{b}	9.130^{b}			
6	1	9.235^{B}	9.206^{B}	9.117^{B}			
	1.5	9.220^{a}	9.146^{a}	9.082^{a}			
	2	9.178^{a}	9.127^{b}	9.056^{a}	65	59	58
	2.5	9.143^{b}	9.079^{b}	9.045^{a}			
	3	9.079^{b}	9.075^{b}	8.977^{b}			

Capital letters (A and B) indicate differences between the numbers of 1 h of incubation and control while the small letters indicate differences between the numbers of current hour and 1 h of incubation at $P<0.05$ based on the LSD test; ^{A,a}No significant difference; ^{B,b}Significant difference; -: No viable cells; Lb2: *L. fermentum*; Lb9: *L. rhamnosus*; Lb10: *L. paracasei*; missing pH (2) and hours, no viable cells

the tolerance of *Lactobacillus* to three bile salts concentrations after 3 h of incubation.

For probiotic bacteria to be active for a long time in the gastrointestinal tract, they should be able to resist administrated antibiotics. In this study, ten antibiotics were used. The results in Figure 2 show that Lb2, Lb9, and Lb10 were highly sensitive to ampicillin, amoxicillin, and erythromycin; this was in accordance with reported by other authors.[32-35] For Lb2, the inhibition zone of ampicillin was higher than that of amoxicillin and erythromycin while the inhibition zones of these three antibiotics were the same for Lb9 while for Lb10, amoxicillin made a higher inhibition zone than other two antibiotics. Lb9 was resistant to trimethoprim-sulfamethoxazole, amikacin, and neomycin where there was no inhibition zone; these results are similar to those of Coppola *et al*.[36]

Table 2: Bile salt tolerance percentages of *Lactobacillus* isolates at different bile salts concentrations

Bile salt concentrations %	h	Mean of viable count (log10 CFU/ml)			Survival percentage		
		Lb2	Lb9	Lb10	Lb2	Lb9	Lb10
0.3	1	9.385^{A}	9.372^{B}	9.245^{B}	70	68	58
	1.5	9.369^{a}	9.332^{a}	9.212^{a}			
	2	9.235^{b}	9.278^{b}	9.133^{b}			
	2.5	9.193^{b}	9.262^{b}	9.130^{b}			
	3	9.146^{b}	9.260^{b}	9.079^{b}			
0.5	1	9.305^{B}	9.324^{B}	9.103^{B}	52	49	43
	1.5	9.176^{b}	9.206^{b}	9.089^{a}			
	2	9.133^{b}	9.146^{b}	9.021^{a}			
	2.5	9.082^{b}	9.082^{b}	8.924^{b}			
	3	8.991^{b}	8.968^{b}	9.004^{a}			
0.7	1	9.170^{B}	9.060^{B}	9.068^{B}	42	30	44
	1.5	9.117^{a}	9.029^{a}	9.060^{a}			
	2	9.045^{b}	8.954^{a}	9.056^{a}			
	2.5	9.029^{b}	8.857^{b}	9.037^{a}			
	3	8.908^{b}	8.851^{b}	9.017^{a}			

Capital letters (A and B) indicate differences between the numbers of 1 h of incubation and control while the small letters indicate differences between the numbers of current hour and 1 h of incubation at $P<0.05$ based on the LSD test; ^{A,a}No significant difference; ^{B,b}Significant difference ($P<0.05$); Lb2: *L. fermentum*; Lb9: *L. rhamnosus*; Lb10: *L. paracasei*

Table 3: Cell surface hydrophobicity of *Lactobacillus* isolates

***Lactobacillus* isolates**	Absorbance		Hydrophobicity percentage
	A0	A	
Lactobacillus fermentum Lb2	0.8273	0.1881	77.26 (high)
Lactobacillus rhamnosus Lb9	0.9965	0.3132	68.56 (medium)
Lactobacillus paracasei Lb10	0.8206	0.2872	65.00 (medium)

A0: Before adding n-hexadecane; A: After adding n-hexadecane; low: 0–35%; medium: 36–70%; high: 71–100%

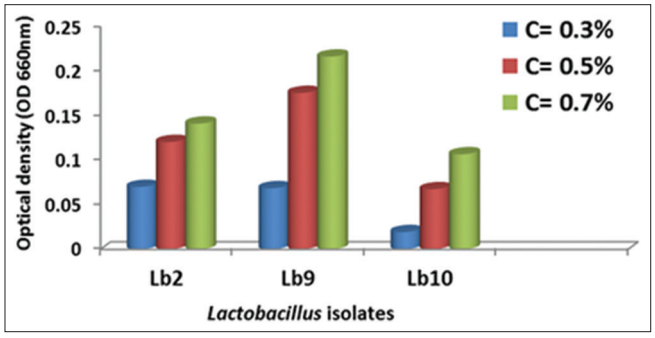

Figure 1: Tolerance of *Lactobacillus* isolates; Lb2, *Lactobacillus fermentum*; Lb9, *Lactobacillus rhamnosus;* and Lb10, *Lactobacillus paracasei* to bile salts concentrations (0.3%, 0.5%, and 0.7%)

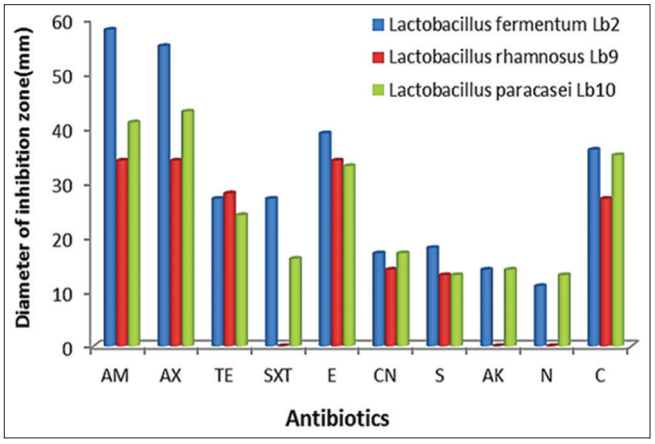

Figure 2: Antibiotics susceptibility test of *Lactobacillus* isolates; AM: Ampicillin; AX: Amoxicillin; TE: Tetracycline; SXT: Trimethoprim-sulfamethoxazole; E: Erythromycin; CN: Gentamycin; S: Streptomycin; AK: Amikacin; N: Neomycin; C: Chloramphenicol

Cell surface hydrophobicity of *Lactobacillus* isolates was determined to correlate the data with their ability to adhere to the intestinal epithelium. Cell surface hydrophobicity was determined using the method of BATH. The percent cell surface hydrophobicity was calculated, as shown in Table 3. According to three levels of hydrophobicity (low: 0–35%, medium: 36–70%, and high: 71–100%) that determined by Ocana *et al.*[37] Lb2 showed a high hydrophobicity which was 77.26% while Lb9 and Lb10 showed medium hydrophobicity with percentage of 68.56% and 65%, respectively, the results of Lb2

and Lb9 are similar to previous study of García *et al*.[35] The BATH test quantifies the surface hydrophobicity of bacteria, and it has widely been used to estimate the physicochemical component in adhesion ability of bacterial strains.[38] Hence, based on the hydrophobicity or the charge of the bacterial outer layer, the adhesion ability to hydrophobic surfaces such as mucus can be characterized indirectly.[39]

However, consumption products that contain probiotic bacteria such as dairy products and fermented vegetables as well as eat healthy foods rich in fibers and prebiotics will contribute to increase the numbers of these useful bacteria in our gastrointestinal tract, and this lead to reestablishment of normal microbiota balance and enhancement their activities.

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CONCLUSION

Depending on the results of this study, the bacterial isolate *L. fermentum* Lb2 showed up good probiotic behavior in respect of acid and bile salt tolerance and adhesion ability to hydrocarbons. However, more studies are required about this isolate and thus could be good candidate to use as probiotic.

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Research Article

Cytotoxic Effects of *Pistacia khinjuk* Seed Extracts on Different Cell Lines and its Mitogenic Effects on Blood Lymphocyte *In Vitro*

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ABSTRACT

Reports indicated that extract *Pistacia khinjuk* has anti-inflammatory, antipyretic, antibacterial, and antiviral, in treating of diarrhea and throat infections and has hepatoprotective effects against acetaminophen and carbon tetrachloride. This study was undertaken to investigate the possible cytotoxic effects of methanolic and aqueous seeds extract of *P. khinjuk* on different tumors (rhabdomyosarcoma [RD] and murine mammary adenocarcinoma [Ahmed-Mohammed-Nahi-2003 (AMN-3)]) and normal cell lines (murine fibroblast) and its mitogenic effects on blood lymphocytes. The cytotoxic effects of *P. khinjuk* seed extracts were evaluated on two tumor cell lines, RD and murine mammary adenocarcinoma (AMN-3) and one normal cell line, murine fibroblast (L20B). Moreover, the mitogenic effects of the plant extract were studied, on human blood lymphocytes. Both methanolic and aqueous seed extracts of *P. khinjuk* significantly induced tumor cell lines and the normal cell line proliferation, especially in highest concentrations. The results show that the extracts induced significant increases in human blood lymphocyte proliferation at 72 h. This activity of plant extracts recommends it as a good mitogenic agent in researches; in conclusion, seed extracts of *P. khinjuk* induced proliferation of all tested cell lines. High concentrations of both aqueous and methanolic seed extracts of *P. khinjuk* showed mitogenic effects.

Keywords: Mitogenic effect, *Pistacia khinjuk*, proliferative effect, tissue culture

INTRODUCTION

Conventionally, *Pistacia khinjuk* used as tonic and expectorant, it has been used in cough, fever, and asthma.[1] Few investigations have been reported the effect of different crude and isolated compound extracts of *P. khinjuk* on different diseases. Some species of *Pistacia* have been used in folk medicine as anti-inflammatory, antipyretic, antibacterial, and antiviral, in treating diarrhea and throat infection.[2] Kordali *et al.* tested the antifungal activity of crude extracts obtained from the leaves of *Pistacia vera*, *Pistacia terebinthus*, and *Pistacia lentiscus*.[3] Ozcelik *et al.* used extracts of P. vera and studied antibacterial, antifungal, and antiviral properties of it, it showed little antibacterial activity, a noticeable antifungal activity, and significant antiviral activity.[4] Ljubuncic *et al.* studied the effects of aqueous extracts prepared from the leaves of *P. lentiscus* in experimental liver disease.[5]

Taran *et al.* (2009) studied the anthelmintic effect of *P. khinjuk* against protoscoleces of *Echinococcus granulosus,* the results of this study indicate *in vitro* anti-echinococcal activity of the essential oil of *P. khinjuk*.[6]

Derwich *et al.* (2010) studied antibacterial activity of the essential oil isolated from the leaf of *P. lentiscus*, the study was conducted to determine the phytochemistry and antibacterial activities of *Pistacia* leaves oil against both Gram-negative and Gram-positive bacteria.[7] In another study, Taran *et al*. investigated the antimicrobial activity of the leaves of *P. khinjuk* and they found that some major constituents of essential oil from the aerial parts of *P. khinjuk* extracts showed antibacterial and antifungal activities.[1] Dizaye examined the effect of aqueous extract of *P. khinjuk* on acetaminophen and carbon tetrachloride-induced acute liver toxicity in albino rats, he concluded that an aqueous extract of *P. khinjuk* has hepatoprotective effects against acetaminophen and carbon tetrachloride.[8] Tohidi *et al.* investigated the antibacterial and wound healing activity in both *P. khinjuk* and *Pistacia atlantica*;

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the results suggested that antibacterial and wound healing activities of *P. khinjuk* are better than *P. atlantica* at the same concentrations.[2]

According to our knowledge, no data are available regarding the cytogenetic and mitogenic activities of *P. khinjuk*. Therefore, this study was designed to investigate the cytogenic effects of methanolic and aqueous seed extract of *P. khinjuk* on two tumor cell lines (rhabdomyosarcoma [RD] and murine mammary adenocarcinoma) and one normal cell line (murine fibroblast). Moreover, the mitogenic effects of *P. khinjuk* was studded on human blood lymphocytes.

MATERIALS AND METHODS

Plant Collection

P. khinjuk were collected from Erbil (Shaqlawa) Governorate. Plants were deposited to be identified, the identification done by the Department of Pharmacognosy, College of Pharmacy of Hawler Medical University, Iraq.

Seeds of *P. khinjuk* were separated and dried at room temperature according to Al-Barazanjy. The dried seeds were grind into powder by electrical grinder (mesh no. 0.5 mm), and the powder was kept in plastic bags in a deep freezer (−20°C) until use.[9]

Preparation of aqueous extracts from P. khinjuk seeds

Aqueous extract was prepared from the grind powder according to Al-Barazanjy. Then, crude dried extract was placed in labeled, tightly sealed plastic tubes, and stored at −20°C until used.[9]

For the following experiments, 1 g of aqueous seeds extract was dissolved into 100 ml phosphate buffer saline (PBS), the suspension then filtered and sterilized using 0.2 μm sterile Millipore filter and kept in a deep freeze in (−20°C) until use.

Preparation of methanolic extracts from P. khinjuk seeds

Methanolic extraction was prepared according to Harborn (1984), then the extract was stored in a tightly sealed plastic test tube at −20°C until use.[10]

Cell Lines

Rhabdomyosarcoma (RD) cell line

This is a human cell line derived from a biopsy specimen obtained from a pelvic rhabdomyosarcoma of a 7-year-old Caucasian girl.[11] Passages 258–263 of RD cell line were used throughout this study and RPMI-1640 was used for maintaining the cells.

Ahmed-Mohammed-Nahi-2003 (AMN-3) cell line

This cell line is a murine mammary adenocarcinoma cell line derived from a spontaneous mammary adenocarcinoma of female BALB/c mice.[12] The cells were maintained in RPMI-1640 medium. Passages128–135 of AMN-3 cell line were used throughout this study.

L20B cell line

This cell line is a murine cell line derived from mouse L cells (fibroblasts) expressing the human poliovirus receptor.[13] Passage numbers (14–18) of the L20B cell line were used in this study and it was maintained in RPMI-1640 medium containing 10% bovine calf serum.

Cytotoxicity Assay

The cytotoxicity protocol was depending on Flick and Gifford, 1984.[14] Cells were plated in a 96-well flat-bottomed plate, after adhesion, serial dilutions of aqueous and methanolic extract separately three replicates for each concentration were placed in three columns for each type (200 μl from each extract) to the appropriate wells and incubated for 24, 48, or 72 h at 37°C, 5–10% CO_2 in a humidified environment. Untreated cells were used as controls. Then, the supernatants were decanted off and cells in each well were washed with PBS twice from, and 50 μl of 0.01% neutral red dye was added to each well and reincubated for 2 h; at the end of incubation, excess dye was removed by washing the wells twice with 150 μl PBS, then 125 μl of extraction dye solution was added.[10] The optical density (OD) of each well was read using an enzyme-linked immunosorbent assay reader at a transmitting wavelength on 492 nm.

Mitogenic Activity of *P. khinjuk* Seeds Extracts *In Vitro*

Human blood culture was prepared by collecting blood sample by vein puncture and transferred into heparinized tubes containing media (RPMI-1640); then, different extracts of *P. khinjuk* were added to blood culture tubes (three tubes for each) instate of polyhydroxyalkanoates (PHA) and three tubes prepared with PHA as control positive and other three without PHA as negative control. All tubes incubate in CO_2 incubator and shacked gently each 15 min at the 1st h, 30 min at the 2nd h, and ones at the 3rd and 4th h then each 12 h and incubated for 48 and 72 h; then, the cells were harvested, slides prepared and stained according to Suman *et al.,* 2006.[15]

Statistical Analysis

Significance level was ascertained by one-way analysis of variance, followed by Student-Newman-Keuls multiple tests. Results were expressed as the mean ± standard error of the mean. $P < 0.05$ was considered statistically significant. All statistical procedures were performed with SPSS software version 16.

RESULTS

Cytotoxic Effects of Aqueous and Methanolic Extract of *P. khinjuk* Seeds on RD Tumor Cell Line

Tables 1 and 2 show the effect of both aqueous and methanolic seed extracts of *P. khinjuk* on RD tumor cell line. The inhibitory effect was found in the proliferation of RD tumor cells when treated with 78.125, 312.5, 625, and 1250 μg/ml of seed aqueous extract for 24 h. However, an increase in the proliferation of RD cells was detected when these cells treated for 24 h with 10,000 μg/ml, in which the OD was 0.4773 ± 0.0115. This induction effect was also detected at 48 h treatment with the same plant extract, in which a significant increase was observed at

Table 1: Cytotoxic effect of aqueous extract of *P. khinjuk* seeds on the growth of RD tumor cell line

Concentrations (μg/ml)	Exposure period		
	24 h	48 h	72 h
Control	0.3987±0.008^{b}	0.1133±0.0003^{a}	0.1127±0.0019^{a}
78.125	0.3567±0.024^{a}	0.1413±0.0032^{b}	0.117±0.0051^{a}
156.25	0.3520±0.0144^{a}	0.1477±0.0067^{bc}	0.1097±0.0082^{a}
312.5	0.3440±0.0016^{a}	0.1467±0.0027^{bc}	0.1157±0.0113^{a}
625	0.3467±0.0043^{a}	0.1467±0.0027^{bc}	0.1233±0.0035^{a}
1250	0.3463±0.0018^{a}	0.1637±0.0059^{bc}	0.1293±0.0027^{a}
2500	0.3997±0.0023^{b}	0.1667±0.0023^{c}	0.132±0.0044^{a}
5000	0.3977±0.0009^{b}	0.1413±0.0032^{c}	0.1277±0.0062^{a}
10,000	0.4773±0.0115^{c}	0.1687±0.0064^{c}	0.1193±0.0049^{a}

P. khinjuk: *Pistacia khinjuk*, RD: Rhabdomyosarcoma. *Similar letters indicate no significant differences. *Different letters indicate significant differences at $P<0.05$

Table 2: Cytotoxic effect of methanolic seeds extract of *P. khinjuk* seeds on the growth of RD tumor cell line

Concentrations(μg/ml)	Exposure period		
	24 h	48 h	72 h
Control	0.1327±0.0101^{a}	0.1470±0.0012^{a}	0.1427±0.004^{ab}
78.125	0.1547±0.0009^{b}	0.1543±0.0022^{a}	0.1704±0.0053^{b}
156.25	0.1637±0.0015^{b}	0.1543±0.0009^{a}	0.1627±0.0067^{ab}
312.5	0.147±0.0052^{b}	0.1540±0.0017^{a}	0.1667±0.0064^{ab}
625	0.1613±0.0018^{b}	0.1533±0.0035^{a}	0.1607±0.0046^{ab}
1250	0.1633±0.0047^{b}	0.1550±0.0038^{a}	0.1547±0.0015^{ab}
2500	0.1633±0.0019^{b}	0.1653±0.0018^{b}	0.1370±0.0131^{a}
5000	0.164±0.0027^{b}	0.1650±0.0012^{b}	0.1533±0.0026^{ab}
10,000	0.1667±0.0009^{b}	0.1690±0.0036^{b}	0.1537±0.0067^{ab}

P. khinjuk: *Pistacia khinjuk*, RD: Rhabdomyosarcoma. *Similar letters indicate no significant differences. *Different letters indicate significant differences at $P<0.05$

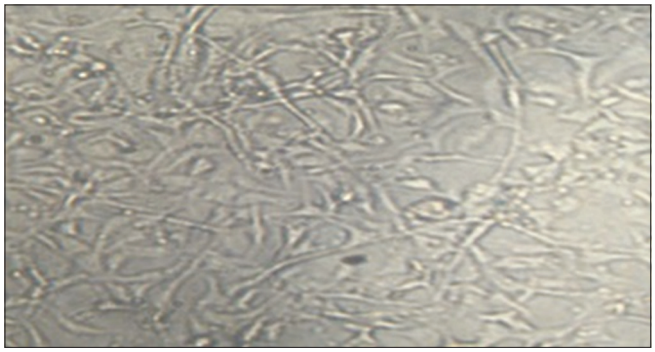

Figure 1: Confluent monolayer of untreated rhabdomyosarcoma tumor cells (×400)

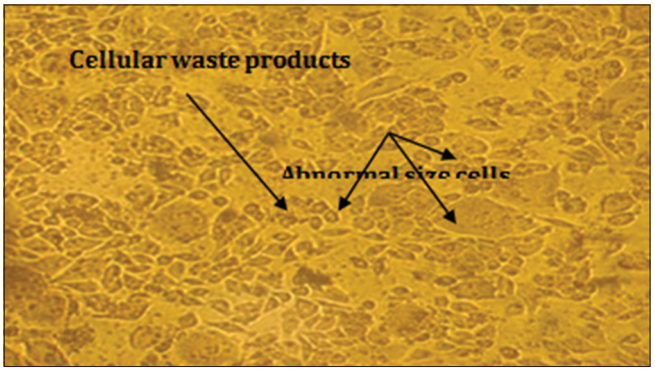

Figure 2: Rhabdomyosarcoma tumor cells treated with 10,000 μg/ml *Pistacia khinjuk* aqueous seeds extract 48 h (×400)

48 h of treatment, while the highest level of proliferation was detected in concentration of 10,000 μg/ml at 48 h of exposure with OD of 0.1687 ± 0.0064 when compared with its control (0.1133 ± 0.0003) [Figure 1]. In contrast, a non-significant difference was observed when this type of cell line was treated with aqueous seed extract of *P. khinjuk* for 72 h in comparison with its control. Whereas the effect of methanolic extract of the same plant on RD tumor cell line was stimulation in growth of the cells at 24 h and 48 h of exposure, particularly at the higher concentrations (5000 μg/ml and 10,000 μg/ml), and the results showed non-significant differences at 72 h of treatment.

As shown in Figures 2 and 3, treatment of the cells with the highest concentrations of both extracts caused remarkable changes in the confluent monolayer appearance.

Cytotoxic Effects of Aqueous and Methanolic Extract of *P. khinjuk* Seeds on AMN-3 Tumor Cell Line

The aqueous extract was more effective against the proliferation of AMN-3 cells at 24 h of treatment than other treatment periods. The treatment for both 48 and 72 h showed non-significant differences in cell proliferation when compared with the control group, but the treatment for 24 h showed significant decreases in the proliferation of AMN-3 cells and the effect was started from 78.125 μg/ml to highest concentration of 10,000 μg/ml, Table 3.

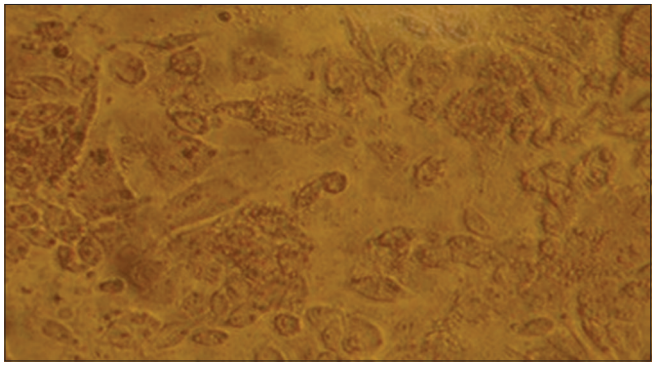

Figure 3: Rhabdomyosarcoma tumor cells treated with 10,000 μg/ml *Pistacia khinjuk* methanolic seeds extract 48 h (×400)

Table 4 shows the effect of methanolic seeds extract of *P. khinjuk* on AMN-3 tumor cells which exhibited a significant increase in proliferation of tested cell line in all concentrations at all periods of treatment, particularly at the highest concentrations of 5000 μg/ml and 10,000 μg/ml.

Figure 4 shows the effect of aqueous seeds extract on AMN-3 cells at 24 h treatment, in which some cells with different changes in their shape and size were seen, the monolayer appearance is absent.

Figure 5 shows the shape and size of AMN-3 cells similar to that of untreated cells in control [Figure 6].

Table 3: Cytotoxic effect of aqueous extract of *P. khinjuk* seeds on the growth of AMN-3 tumor cell line

Concentrations(μg/ml)	Exposure period		
	24 h	48 h	72 h
Control	0.3190±0.2355^{a}	0.1030±0.0089^{a}	0.2267±0.0034^{a}
78.125	0.1027±0.0029^{b}	0.1080±0.0027^{a}	0.2097±0.0018^{a}
156.25	0.0963±0.0015^{b}	0.1067±0.0015^{a}	0.2233±0.0150^{a}
312.5	0.1067±0.0038^{b}	0.0960±0.0025^{a}	0.2303±0.0064^{a}
625	0.1060±0.0051^{b}	0.1070±0.0031^{a}	0.2594±0.0091^{a}
1250	0.1103±0.0060^{b}	0.1062±0.0029^{a}	0.2920±0.0089^{a}
2500	0.1160±0.0035^{b}	0.1340±0.0031^{a}	0.2910±0.0035^{a}
5000	0.1097±0.0019^{b}	0.1270±0.0038^{a}	0.2930±0.0076^{a}
10,000	0.1137±0.0023^{b}	0.1113±0.0033^{a}	0.2937±0.0033^{a}

*Similar letters indicate no significant differences. *Different letters indicate significant differences at $P<0.05$. *P. khinjuk*: *Pistacia khinjuk*, AMN-3: Ahmed-Mohammed-Nahi-2003

Table 4: Cytotoxic effect of methanolic extract *P. khinjuk* seeds on the growth of AMN-3 tumor cell line

Concentrations (μg/ml)	Exposure period		
	24 h	48 h	72 h
Control	0.0820±0.0025^{a}	0.1030±0.0089^{a}	0.2267±0.0034^{a}
78.125	0.1007±0.0029^{b}	0.1133±0.0026^{ab}	0.2277±0.0069^{a}
156.25	0.0957±0.0020^{b}	0.1210±0.0012^{abc}	0.2307±0.0113^{ab}
312.5	0.1023±0.0038^{b}	0.1217±0.0020^{bc}	0.2410±0.0164^{abc}
625	0.1027±0.0033^{b}	0.1207±0.0035^{bc}	0.2443±0.0085^{abc}
1250	0.1020±0.0015^{b}	0.1347±0.0037^{c}	0.2823±0.0033^{abc}
2500	0.0997±0.0012^{cd}	0.1540±0.0049e	0.2990±0.0006^{abc}
5000	0.1093±0.0018^{d}	0.1367±0.0024^{d}	0.3040±0.0025^{bc}
10,000	0.1103±0.0015^{c}	0.1160±0.0065^{c}	0.3093±0.0064^{c}

P. khinjuk: *Pistacia khinjuk*, AMN-3: Ahmed-Mohammed-Nahi-2003. *Similar letters indicate no significant differences. *Different letters indicate significant differences at $P<0.05$

Table 5: Cytotoxic effect of aqueous extract of *P. khinjuk* seeds on the growth of L20B cell line

Concentrations (μg/ml)	Exposure period		
	24 h	48 h	72 h
Control	0.0947±0.0013[a]	0.1±0.0061[a]	0.0993±0.0003[d]
78.125	0.0940±0.0027[a]	0.1±0.0015[a]	0.0707±0.0012[a]
156.25	0.0947±0.0032[a]	0.1003±0.0009[a]	0.0777±0.0007[b]
312.5	0.0957±0.0039[a]	0.1030±0.0006[a]	0.0797±0.0007[b]
625	0.960±0.0031[a]	0.1030±0.0006[a]	0.003±0.0019[b]
1250	0.0963±0.0017[a]	0.1077±0.0032[a]	0.0777±0.0019[b]
2500	0.0940±0.0015[a]	0.1083±0.0015[a]	0.0887±0.0032[c]
5000	0.1140±0.0135[a]	0.113±0.0007[a]	0.1030±0.0025[d]
10,000	0.1153±0.0019[a]	0.1753±0.0058[b]	0.0910±0.0021[c]

*Similar letters indicate no significant differences.*Different letters indicate significant differences at $P<0.05$. *P. khinjuk*: *Pistacia khinjuk,*

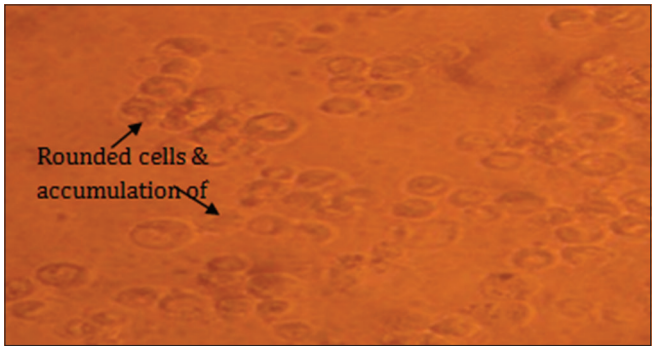

Figure 4: Ahmed-Mohammed-Nahi-2003 tumor cells treated with 10,000 μg/ml of *Pistacia khinjuk* aqueous seeds extract at 24 h of exposure (×400)

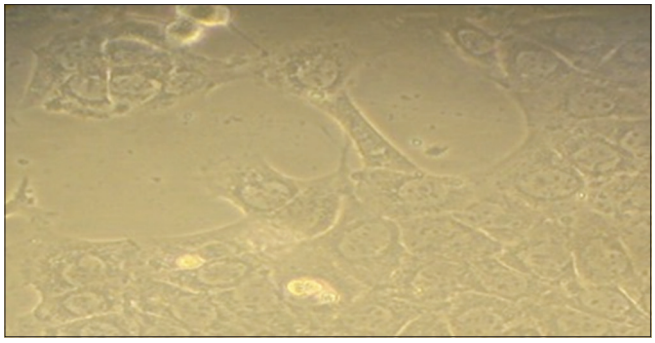

Figure 5: Ahmed-Mohammed-Nahi-2003 tumor cells treated with 5000 μg/ml of *Pistacia khinjuk* methanolic seeds extract at 72 h of exposure (×400)

Cytotoxic Effects of Aqueous and Methanolic Extract of *P. khinjuk* Seeds on L20B Cell Line

Table 5 shows the effect of *P. khinjuk* aqueous seeds extract on the growth of L20B cells. During the first 2 days of incubation, the aqueous extract did not stimulate the growth of L20B cells as compared with control [Figure 7].

Only a significant induction of cell proliferation was detected when treated with 10,000 μg/ml for 48 h of exposure. After 72 h of treatment, the aqueous extract significantly

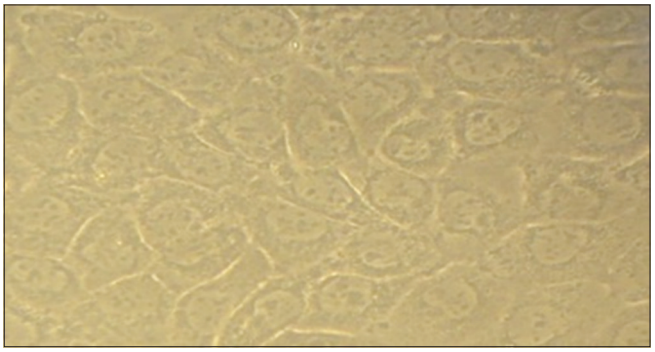

Figure 6: Confluent monolayer of untreated Ahmed-Mohammed-Nahi-2003 tumor cells (×400)

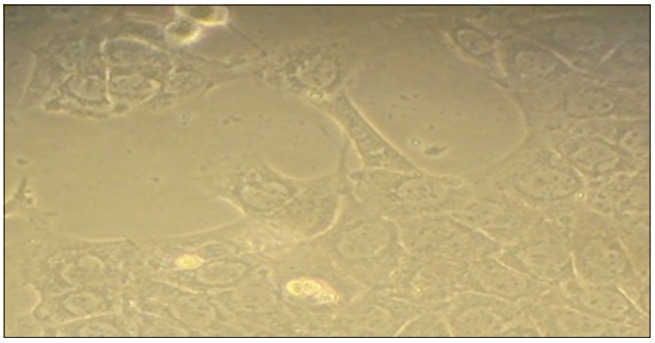

Figure 7: Confluent monolayer of untreated L20B cells (×400)

inhibited cell growth at almost all concentrations. This effect is shown in Figure 8, in which the cells exhibited different changes in cell shape. Some rounded, binucleated, and multinucleated cells were detected when treated with 10,000 μg/ml.

Viability of L20B cells after treatment with the methanolic extract is shown in Table 6. Treatment of cells with concentrations of 2500 μg/ml and 10,000 μg/ml for 24 h caused a significant increase in cell viability. All the other concentrations have no significant effects on cell viability. Non significant differences were detected when the cells treated with78.125, 156.25, 312.5, and 625 μg/ml at 48 h, while the cells when treated with higher concentrations 1250, 2500, 5000, and 10, 000μg/ml at 48h a significant increases were

detected in cell viability. The results also showed insignificant differences at 72 h of exposure.

Figure 9 shows the induction effect of this extract 10,000 μg/ml for 48 h on L20B cells with different changes such as rounded and binucleated and multinucleated cells.

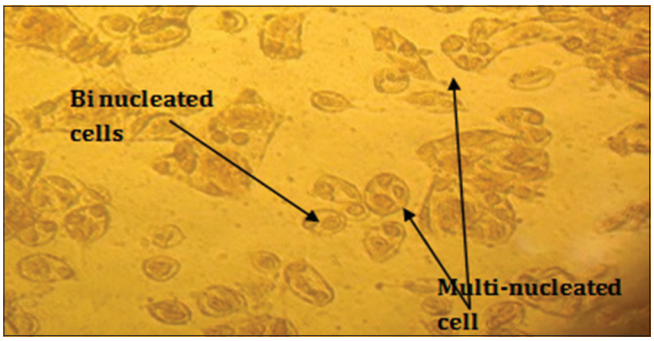

Figure 8: L20B cells treated with 10,000 μg/ml of *Pistacia khinjuk* aqueous seeds extract at 72 h of exposure (×400)

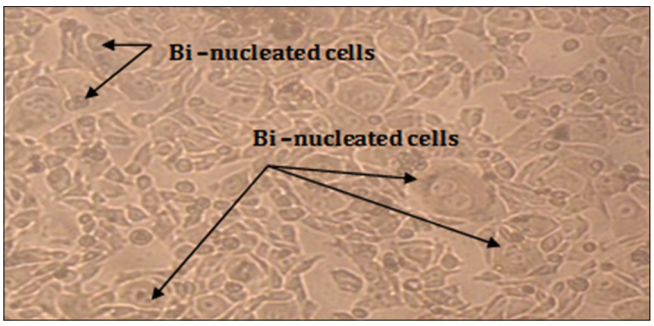

Figure 9: L20B cells treated with 10,000 μg/ml of *Pistacia khinjuk* methanolic seeds extract at 48 h (×400)

Mitogenic Activity of Aqueous and Methanolic Extracts of *P. khinjuk* on Human Blood Lymphocyte

Aqueous and methanolic extracts of seeds of *P. khinjuk* 10,000 μg/ml were used to detect its effect as mitogen in blood lymphocyte cultures for both 48 and 72 h exposure; the results were compared with positive control group which was treated with PHA as a mitogen and also were compared with the negative control group (group that treated with PBS).

Significant induction was detected in all treated group with 10,000 μg/ml *P. khinjuk* seeds extract for 48 h when compared with negative control group, and non significant differences was detected when the cells treated with aqueous and methanolic extracts for 72 h in all groups when compared with positive control group, but a significant induction was recorded in Mitotic Index (MI) of all groups when compared with the negative control group, Table 7.

DISCUSSION

Effects of Aqueous and Methanolic Seeds Extracts of *P. khinjuk* on RD, AMN-3, and L20B Cell Lines

When both cell lines (RD and AMN-3) treated with seed extract of *P. khinjuk,* a significant increases in cell proliferation were detected for both aqueous and methanolic extracts, except exposure of both types of cell lines for 72h to aqueous extract, this increase might be due to the fact that extractions from some herbs can restrain cancer cells and induce its proliferation. Cao *et al.* estimated that extractions from some herbs can restrain hepatocarcinoma, and the herbal extractions of flavonoid and arsenic trioxide cannot only

Table 6: Cytotoxic effect of methanolic extract of *P. khinjuk* seeds on the growth of L20B cell line

Concentrations (μg/ml)	Exposure period		
	24 h	48 h	72 h
Control	0.1±0.0061^{a}	0.1001±0.0061^{a}	0.993±0.0003^{ab}
78.125	0.1±0.0012^{a}	0.1003±0.0067^{a}	0.0913±0.0017^{a}
156.25	0.1003±0.0019^{a}	0.1003±0.0049^{a}	0.0920±0.0006^{a}
312.5	0.1127±0.0029^{a}	0.1027±0.0024^{a}	0.0937±0.0026^{a}
625	0.1113±0.0009^{a}	0.1323±0.0024^{a}	0.1037±0.0047^{ab}
1250	0.1270±0.0050^{a}	0.2417±0.0197^{d}	0.1033±0.0018^{ab}
2500	0.1777±0.0112^{c}	0.2430±0.0103^{bd}	0.1120±0.0027^{b}
5000	0.1317±0.0038^{a}	0.2073±0.0049^{d}	0.0910±0.0012^{b}
10,000	0.1550±0.1617^{b}	0.2977±0.0089^{e}	0.1030±0.0060^{ab}

*Similar letters indicate no significant differences. *Different letters indicate significant differences at $P<0.05$. *P. khinjuk*: *Pistacia khinjuk*

Table 7: Mean±SEM for mitotic index of human blood lymphocyte when *P. khinjuk* used as mitogenic factor

Exposure period	Positive control (PHA)	Negative control (PBS)	*P. khinjuk* methanolic seeds extract 10,000 μg/ml	*P. khinjuk* aqueous seeds extract 10,000 μg/ml
48 h treatment	0.4893±0.00445^{a}	0.2438±0.00185^{d}	0.3564±0.02775^{c}	0.3767±0.00845^{c}
72 h treatment	0.4906±0.00561^{a}	0.2862±0.29093^{b}	0.3822±0.00740^{a}	0.3807±0.00491^{a}

*Similar letters indicate no significant differences. *Different letters indicate significant differences at $P<0.05$. *P. khinjuk*: *Pistacia khinjuk,* PHA: Polyhydroxyalkanoates, PBS: Phosphate buffer saline, SEM: Standard error of the mean

restrain the proliferation of hepatocarcinoma cells but also induce apoptosis of hepatocarcinoma cells.[16]

Rui-Chuan *et al*. reported that Chinese medicine can induce cytodifferentiation of hepatocarcinoma, also rhubarb acid could restrain cell proliferation of various tumors such as mastocarcinoma, lung cancer, hepatocarcinoma, and colon carcinoma, and has synergistic effects when used in combination with Mutamycin.[17] It could inhibit synthesis of DNA, promote apoptosis of cancer cells, and also it has been found that the inhibitory effect of repression of rhubarb on cancer cells is closely related with cell signal transduction.[18]

Tohidi *et al*. and Azadpour *et al.* found that the extract of *P. khinjuk* showed faster healing compared with control groups, faster wound contraction rate may be due to the presence of flavonoids, which is responsible for the free radical scavenging activity that is believed to be one of the most important components of wound healing that also be an evidence for the increased number of cells when treated with *P. khinjuk* extracts which may be caused rearrangement of the cell and induction in there proliferation by the presence of some of those phytochemical compounds such as flavonoids.[2,19]

In general, oleic and linoleic acids are known for their anti-inflammatory properties. Linoleic and alpha-linoleic acid provide lipids necessary for cell membrane repair and cellular respiration.[20]

Tavakoli and Pazhouhanmerhr found that *P. khinjuk* fruits when analyzed contain linoleic and linoleic acid, and the presence of these two types of essential fatty acids may be caused induction in cell proliferation when used for cell membrane repair.[21]

Moulos *et al.,* 2009, demonstrated that exposure of Lewis lung carcinomas to mastic oil (mastic oil from *P. lentiscus*) caused a time-dependent alteration in the expression of 925 genes.[22]

Mirian *et al*., 2015, found that after the exposure of cell lines to methanolic extracts of three plants, the cells shows high survival when they treated with P. khinjuk extract in comparison with other two extracts, and this property may be due to the presence of essential oils.[23]

The effect of those extracts on L20B normal cells was also an induction of cell proliferation at high concentrations of all types of extracts and this induction in currently applied extract, lacks selectivity, and does not spare normal (non-malignant) cells from cancer cells, it also often induces resistance of tumor cells to antineoplastic agents, generally in the form of "multidrug resistance" to structurally unrelated compounds.[24]

Mitogenic Activity of Aqueous and Methanolic Extracts of *P. khinjuk* Seeds on Human Blood Lymphocyte

Aqueous and methanolic extracts from both leaves and seeds of *P. khinjuk* were tested as a mitogen on blood lymphocyte. The results of the present study showed non-significant differences were found between the values of MI of each human lymphocyte that treated for 72 h either by aqueous or methanolic seeds extracts of *P. khinjuk* and positive control group (PHA), the same results were obtained after the treatment with methanolic leaves extract for 48h. These results indicated that the activity of all these extracts is similar to that of PHA.

The increase in a number of cells when treated with *P. khinjuk* extracts may be caused by rearrangement of the cells and induction in their proliferation according to the presence of some phytochemical compounds such as flavonoids.[2]

Tavakoli and Pazhouhanmerhr found that *P. khinjuk* fruits when analyzed contain linoleic and linoleic acid and the presence of these two types of essential fatty acids may be caused induction in cell proliferation when used for repairing of the cell membrane.[21]

Ghosh and Gaba mentioned that *P. khinjuk* fruits and leaves have wound healing properties which may be due to the presence of different phytochemical compounds in these extracts.[25]

CONCLUSIONS

Methanolic and aqueous seed extracts of *P. khinjuk* induced proliferation of all tested cell lines. High concentrations of both aqueous and methanolic seed extracts of *P. khinjuk* showed mitogenic effects.

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Research Article

Levels of Apelin, Endoglin, and Transforming Growth Factor Beta 1 in Iraqi Women with Polycystic Ovary Syndrome

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ABSTRACT

Polycystic ovary syndrome (PCOS) is one of the most common causes of infertility in women of reproductive age. The aim of the study was to determine the level of apelin, insulin resistance (IR), transforming growth factor beta 1 (TGF-β1), and endoglin in women with polycystic ovary syndrome. Fifty PCOS patients and 40 non-PCOS infertile patients were recruited. The fasting serum levels of folliclestimulating hormone (FSH), luteinizing hormone (LH), testosterone (T), prolactin, fasting blood glucose, insulin, and apelin at the early follicular phase were measured. Levels of apelin, LH, LH/FSH, T, and fasting insulin, as well as homeostatic model assessment of IR (HOMA-IR) in PCOS patients, were significantly higher than in the control group. Correlation analysis showed that apelin level was positively correlated with body mass index and HOMA-IR. Apelin levels and TGF-β1 were significantly increased in PCOS patients while show decrease levels of endoglin.

Keywords: Apelin, endoglin, transforming growth factor beta

INTRODUCTION

Polycystic ovary syndrome (PCOS) is the most common endocrine disease affecting women of reproductive age. The prevalence of PCOS varies according to the diagnostic criteria used, with estimates ranging from 9% in women of reproductive age, according to NIH criteria, up to 18%, with the Rotterdam criteria.[1]

It is primarily characterized by ovulatory dysfunction and hyperandrogenism,[2] but the clinical presentation is heterogeneous and patients may present some of various signs and symptoms. This heterogeneity seems to be modulated by multiple factors such as prenatal androgen exposure, nutritional status in the uterus, genetic factors, and ethnicity, insulin resistance (IR) of puberty and/or exaggerated adrenarche and changes in body weight.[3] Environmental factors, such as obesity, appear to exacerbate the underlying genetic predisposition. Concerning ethnicity, the presence of hirsutism is less frequent in Asian patients (around 10%), compared to Caucasian ones (70%).[4]

Obesity is a prevalent characteristic of PCOS, ranging from 12.5% to 100%, with a pooled estimated prevalence of 49%, as shown by a recent meta-analysis.[5] The presence of obesity may exacerbate the metabolic and reproductive disorders associated with the syndrome, including IR, dyslipidemia, and metabolic syndrome.[6] A meta-analysis[7] has shown that women with PCOS have higher levels of triglycerides (TG), low-density lipoprotein (LDL)-cholesterol and total cholesterol (TC), and lower high-density lipoprotein (HDL)-cholesterol levels compared with control women, regardless of body mass index (BMI). In addition, PCOS women present higher risk for type 2 diabetes. PCOS is also associated with a clustering of cardiovascular risk factors.[8] However, there is no definitive evidence for increased cardiovascular events, nor data showing that PCOS alone leads to increased cardiovascular risk independent of associated risk factors. In fact, more rigorous cohort studies of long-term cardiovascular outcomes and clinical trials of risk factor modification are required for women with PCOS.

In addition, evidence suggests clinical phenotypes are related with different metabolic risks. In this sense, IR seems to be a specific feature of the classic phenotype and, to a lesser extent, of the ovulatory phenotype. Non-hyperandrogenic phenotype behaves as a separate group that is metabolically similar to non-PCOS women.[7]

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Adipose tissue is considered not only a storage tissue but also aproper endocrine organ, metabolically active.[6] IR is considered the main pathogenic factor in the background of increased metabolic disturbances in women with PCOS which can explain hyperandrogenism, menstrual irregularity, and other metabolic manifestations seen in this disease.[7] However, it is not a diagnostic criterion for PCOS.[8]

Obesity alters the expression, circulating levels, and signaling mechanisms of adipose-secreted factors and these changes have been linked to the development of IR, type 2 diabetes, dyslipidemia, atherosclerosis, cancer, and other diseases.[9]

Apelin is an endogenous cytokine,[10] an adipokine,[11] a regulatory peptide,[12] and a neuropeptide[13] that has been linked to obesity and IR.

Apelin is a 36-amino acid peptide (AP-36) generated from a larger precursor the 77-amino acid proapelin. The human preproapelin gene is located on chromosome X at locus Xq25-q26.1.[10]

Collected data from both the clinical and basic settings show that AP (1) correlates with states of IR and obesity, (2) stimulates glucose utilization, (3) decreases insulin secretion, and (4) negatively regulates catecholamine-mediated lipolysis. AP seems to be a key regulator in glucose and lipid metabolism and may be associated with IR and possibly implicated in PCOS which is known to be associated with increased IR[14] and from hence the possible link to apelin levels.

Endoglin (sEng)/CD105 is a transmembrane auxiliary receptor for transforming growth factor beta (TGF-b) it works as a non-signaling coreceptor of the TGF-b modulating its responses. There are a number of reports suggesting that soluble endoglin may be regarded as a biomarker of endothelial dysfunction, for instance, atherosclerosis, hypercholesterolemia,[15] Type 2 diabetes mellitus (T2DM), and hypertension.[16]

TGF-b1 is a multifunctional cytokine that is produced by a variety of cells and its level dysregulated in women with PCOS, which might play a role in the pathophysiology of this syndrome. Substantial evidences implicate that TGF-b1 activation is closely associated with endothelial dysfunction and hypertension.[17] There is scattered evidence for the association between TGF-b1, visceral adiposity, metabolic syndrome, nonalcoholic fatty liver disease, and T2DM.[15]

METHODS

This case–control study was conducted at the obstetrics and gynecology department in medical city in Baghdad. It was conducted on ninety women during the period from March 2018 to September 2018. These were divided into two groups: Group I: Fifty overweight and obese women with PCOS (PCOS group) and Group II: Forty overweight and obese women without PCOS (non-PCOS group). The recruited women were counseled and written informed consent was obtained from each woman before her participation in the study.

Diagnosis of PCOS was made according to the Rotterdam criteria[18] in the presence of at least two of the following: (a) Oligomenorrhea and/or anovulation, (b) biochemical and/or clinical hyperandrogenism, and (c) ultrasound appearance of polycystic ovaries (multiple cysts >12 in number of 2–9 mm size),[3] BMI P 25 kg/m^2.

Samples were obtained between days 2 and 5 of normal spontaneous or withdrawn menstrual cycle. Blood allowed to clot for 10–20 min at room temperature and centrifuged at (2000–3000 RPM) for approximately 20 min to separate the serum from the cells.

2 ml serum was stored at _20_c until the time of AP-36 and hormonal profile assay. Hormonal evaluation included a baseline plasma determination of luteinizing hormone (LH), follicle-stimulating hormone (FSH), dehydroepiandrosterone sulfate, TSH, E2, and free testosterone. All hormones were measured by radioimmunoassay methods using commercial kits (DSL Inc., Webster, Texas, USA). The fasting insulin was measured by radioimmunoassay methods using commercial kits (the intra and inter-assay coefficients of variation were 1.98% and 0.84%, respectively) (Immunospect Corporation, Owensmouth Ave, Canoga Park, CA). Fasting glucose was measured by the glucose oxidase method (Yellow Springs Instrument, Yellow Springs, OH, USA). TC, TG, and HDL were assayed using fully automated clinical chemistry autoanalyzer system Konelab 20i LDL was calculated according to Friedewald formula: LDL = TC−(TG/5+HDL).[19] IR was calculated by homeostatic model assessment (HOMA) using the following formula: HOMA-IR = ([FG in mg/dL_0.05551]_ FI in mU/ml)/22.5. A diagnosis of IR established for HOMA-IR values >2.71 nmol_mU/l[20] equal (FG in mg/dL_FI in mU/ml)/405. IR was diagnosed according to the FG to FI ratio. If a participant's FG/FI was 4.5 or less, she would be classified as being IR and if her FG/FI was >4.5, she would be classified as being non-IR.[21]

RESULTS

The results obtained are shown in Table 1 and Table 2.

Table 1: The mean and SD of biochemical and immune markers level in the patients group and healthy control group

The group	Mean±SD		*P*-value
	PCOS	Control	
Age (year)	28.28±5.11	33.27±6018	0.00033*
BMI (kg/m^2)	32.16±2.28	29.07±1.40	0.00001*
Hirsutism score	13.79±1.2	5.33±0.75	0.00001*
TGF-β1 (ng/ml)	19.34±2.73	12.29±1.19	0.00001*

(Contd...)

Table 1: ***(Continued)***

The group	Mean±SD		*P*-value
	PCOS	Control	
s-endoglin ng/ml	3.37±1.25	5.02±1.34	0.00001*
Apelin (pg/ml)	220.92±16.87	367.05±37.87	0.00001*
Fasting plasma glucose (mg/dl)	94.76±20.71	90.0±14.7	0.00001*
Insulin (IU/ml)	18.76±4.33	12.0±6.5	0.00001*
HOMA	2.44±1.16	1.72±1.01	0.001375*
Prolactin (pg/ml)	13.71±1.25	13.14±1.56	0.02867*
FSH (mIU/ml)	7.04±1.13	6.03±1.32	0.000099*
LH (mIU/ml)	12.13±1.77	5.88±1.2	0.00001*
SHBG (nmol/L)	30.25±2.51	54.12±4.3	0.00001*
DHEA-s (mg/ml)	2.21±0.59	0.95±0.3	0.00001*
Total testosterone (ng/ml)	0.91±0.41	0.59±0.22	0.000017*
Free testosterone (pg/ml)	2.65±0.66	1.064±0.26	0.00001*
h-CRP (μg/ml)	5.75±1.34	2.3±0.7	0.00001*
Cholesterol mg/dl	172.6±49.9	166.0±27.3	0.254423 NS
Triglyceride (mg/dl)	155.86±34.69	103.0±32.2	0.00001*
HDL (mg/dl)	39.14±10.09	39.92±7.15	0.347843 NS
LDL (mg/dl)	103.42±36.31	101.9±24.2	0.413274 NS

*($P<0.05$), **($P<0.01$), NS: Non-significant. PCOS: Polycystic ovary syndrome, BMI: Body mass index, TGF: Transforming growth factor, HOMA: Homeostatic model assessment, FSH: Follicle-stimulating hormone, LH: Luteinizing hormone, SHBG: Sex hormone-binding globulin, DHEA: Dehydroepiandrosterone, h-CRP: High sensitivity C-reactive protein, HDL: High-density lipoprotein, LDL: Low-density lipoprotein

Table 2: Correlation between apelin, transforming growth factor beta, and endoglin with other parameters in the PCOS disease patients

The group	Correlation coefficient (r)		
	TGF-β1 (ng/ml)	s-endoglin ng/ml	Apelin (pg/ml)
Age (year)	0.0917	−0.0948	−0.1572
BMI (kg/m^2)	0.3333	0.0379	0.0353
Hirsutism score	−0.2653	0.0955	−0.1134
TGF-β1 (ng/ml)	-	0.2007	0.1903
s-endoglin ng/ml	0.2007	-	−0.0502
Apelin (pg/ml)	0.1903	−0.0502	-
Fasting plasma glucose (mg/dl)	0.1447	−0.0654	−0.0531
Insulin (IU/ml)	−0.0978	−0.0906	0.0132
HOMA	0.1547	−0.1586	0.1233
Prolactin (pg/ml)	−0.1503	0.3305	−0.223
FSH (mIU/ml)	−0.0841	−0.0611	−0.1269
LH (mIU/ml)	−0.0413	0.0798	0.2071
SHBG (nmol/L)	0.2081	−0.189	0.198
DHEA-s (mg/ml)	−0.1289	−0.0002	−0.1991
Total testosterone (ng/ml)	0.0887	−0.1985	−0.0872
Free testosterone (pg/ml)	0.1543	−0.4447	0.0568
h-CRP (μg/ml)	0.0484	−0.1559	−0.0966
Cholesterol mg/dl	−0.1114	0.0285	0.323
Triglyceride (mg/dl)	0.354	0.1584	0.0839
HDL (mg/dl)	−0.1063	0.1887	−0.0182
LDL (mg/dl)	−0.1063	−0.1289	0.43

*($P<0.05$), **($P<0.01$), NS: Non-significant. PCOS: Polycystic ovary syndrome, BMI: Body mass index, TGF: Transforming growth factor, HOMA: Homeostatic model assessment, FSH: Follicle-stimulating hormone, LH: Luteinizing hormone, SHBG: Sex hormone-binding globulin, DHEA: Dehydroepiandrosterone, h-CRP: High sensitivity C-reactive protein, HDL: High-density lipoprotein, LDL: Low-density lipoprotein

DISCUSSION

PCOS is one of the most common causes of infertility in women of reproductive age. IR is a main pathophysiologic feature in these patients.[2] Apelin (APLN) is a recently discovered adipokine involved in the regulation of various metabolic functions. Its receptor, APLNR, is expressed in reproductive tissues; however, its role in human ovarian cells is unknown. PCOS patients are more vulnerable to develop diabetes, cardiovascular diseases, and metabolic syndrome.[3] IR is prevalent in women with PCOS independently of obesity and is critically involved in reproductive and metabolic complications of the syndrome.[10]

Several tests have been developed to measure IR, some very reliable but complex like the hyperinsulinemic euglycemic glucose clamp and others less precise but easier and less invasive like HOMA-IR.[22] New markers are needed to reach a more reliable assessment of IR.

To date, several surrogate markers have been proposed in literature to facilitate and improve the determination of IR. Many new proteins are strongly involved with PCOS physiopathology and IR such as some adipocytokines (adiponectin, visfatin, vaspin, and apelin), copeptin, irisin, PAI-1, and zonulin. Many other proteins have been proposed as potential new markers of IR in PCOS such as resistin, leptin, retinol-binding protein 4 (RBP4), kisspeptin, and ghrelin, but their role is still controversial.[14]

Adipose tissue is implicated in the secretion of several hormones such as adiponectin, resistin, leptin, visfatin, apelin, and RBP4 called adipocytokines that are involved in energy homeostasis and metabolism.[11]

Apelin is a peptide isolated from bovine stomachs, but it is expressed in several other organs and also in visceral and subcutaneous tissues.[4] In our present study, however, serum apelin levels are significantly higher in PCOS women compared with controls. Moreover, we also observed that apelin level is significantly and positively correlated with BMI and HOMA-IR. Apelin was found to be higher in PCOS patients by Gören *et al*.[23] but without a significant correlation with HOMA-IR. Olszanecka-Glinianowicz *et al*.[18] reported an inverse association between apelin and glucose, insulin, and HOMA-IR values, supporting the role of apelin in the regulation of insulin sensitivity. Apelin levels were higher in non-obese PCOS patients, suggesting a compensatory mechanism for metabolic consequences of IR. Lower serum concentrations of apelin were found in PCOS subjects by Altinkaya *et al*.[19] with positive correlation with BMI, insulin, HOMA-IR, TG, and free testosterone, speculating that apelin can be used as a marker for insulin sensitivity. Conversely, Sun *et al*.[20] observed significantly enhanced apelin concentration in PCOS patients with positive association with BMI and HOMA-IR; treatment with drospirenone-ethinylestradiol plus metformin improved IR and apelin levels. Discrepant findings among the published studies may be attributed to the differences in ethnicity, age, study design, sample size, genetic characteristics of populations, and assessment methodology.

The increase of LH level probably plays an important role in the pathological mechanism of the higher androgens production in the ovaries, which can interfere with the maturation of the oocyte.[21] In the present study, the serum LH levels are significantly higher, while FSH level is lower in PCOS women compared with controls. These results further confirm that high LH level and relative insufficiency of FSH are the characteristics of PCOS.

In line with our results, Tal *et al*. found increased TGF-b1 and decreased sEng in PCOS. These data suggest that increased TGF-b1 bioavailability may contribute to the pathogenesis of PCOS and its increased risk for ovarian hyperstimulation.[17] By contrast, the study conducted by Irani *et al*. found that sENG protein concentration was not different between PCOS and non-PCOS control group at baseline.[15] Women with PCOS have abnormal increase in TGF-b1 bioavailability (TGF-b1/sENG) due to an increase in serum TGF-b1 and decrease in sENG levels.[17]

CONCLUSION

Polycystic ovary syndrome (PCOS) is a condition that affects a woman's hormone levels.

Levels of TGF β1, LH, LH/FSH, T, and, as well as homeostatic model assessment of IR (HOMA-IR) in PCOS patients, were significantly higher than in the control group, while show decrease levels of Apelin and endoglin levels.

Therefore the correlation analysis showed that apelin level was negatively correlated with body mass index (BMI) and HOMA-IR.

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Research Article

Investigation of the Zinc Oxide Nanoparticles Effect on Thyroid and Testosterone Hormones in Male Rats

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ABSTRACT

Exposure to zinc oxide nanoparticles (ZnO NPs) has been increasing steadily, causing more attention being paid to their potential toxicity, including cytotoxicity and genotoxicity. Hence, this study aimed to investigate the effect of ZnO NPs on thyroid hormone triiodothyronine (T3), thyroxine (T4), and thyroid-stimulating hormone (TSH) as well as testosterone hormone in male adult rats. A total of 54 Sprague-Dawley albino adult male rats were divided into nine groups each of six rats, daily treated intraperitoneal with ZnO NPs two different doses (30 and 60) mg/kg in three different periods of time (7, 14, and 28) days, as following: Control groups (Groups 1, 2, and 3): Respectively received intraperitoneal injection with distilled water for 7, 14, and 28 days, experimental groups (Groups 4, 5, and 6): They were rats, respectively, received intraperitoneal dose (60 mg/kg) of ZnO NPs for (7, 14, and 28) days, and group (7, 8, and 9) experimental groups were rats, respectively, received intraperitoneal dose (30 mg/kg) of ZnO NPs for (7, 14, and 28) days. Data showed high significant decrease ($P < 0.01$) in level of T3, T4, TSH, and level of testosterone also decrease at high and low dose for 7, 14, and 28 days.

Keywords: T3, T4, testosterone, thyroid-stimulating hormone, zinc oxide nanoparticles

INTRODUCTION

Zinc oxide nanoparticles (ZnO NPs) are inorganic compounds, called multifunctional material due to unique chemical and physical properties.[1] Zinc is an essential metal it is an activator for more than 300 enzymes in the body.[2] ZnO NPs are one of most widely used in cosmetics and sunscreen because of their efficient UV absorption properties, they are used due to the antimicrobial properties in food packaging.[3] They are also being explored for their potential use as fungicides in agriculture.[4] As well ZnO nanoparticles are used in anticancer drugs and in biomedical applications.[5] Commonly considered to be a material with low toxicity because the ZnO is an essential trace element in human body and is existing in food or added as nutritional complement, so zinc attracts little care during assessment of toxicity of NPs.[6]

ZnO NPs can induce the formation of reactive oxygen species (ROS) that disrupt intracellular metabolic activities and the antioxidant system; these alterations permit generated ROS to interact with and damage DNA, lipids, carbohydrates, and proteins.[7] Thyroid gland is one of major endocrine glands of the body responsible of creating the hormones, thyroxin, and triiodothyronine which are essential for the proper organism development in particular for the nervous system and heart, normal growth, and skeletal maturation.[8] It is considered one of the main regulators of biological processes, during development, and childhood.[9] Decrease in the production of thyroid hormones means hypothyroidism.[10] Many evidence suggesting the role of zinc in the formation and function of thyroid hormones.[11] ZnO NPs cause higher levels of oxidative stress, resulting in inflammation, and cell toxicity.[12] Man testis has two main functions, the production of testosterone and the production of male germ cells, maintenance of spermatogenesis process needs testosterone, the androgen production is regulated by luteinizing hormone, while follicle-stimulating hormone is critical for initiation and maintenance of spermatogenesis.[13,14] Testosterone is the main androgenic hormone in males, it is largely produced by the Leydig cells of the testes, ZnO NPs were internalized by Sertoli cells and Leydig cells resulted in cytotoxicity in a time and dose-dependent manner through the induction of apoptosis, caused by increase in ROS related with loss of

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mitochondrial membrane potential, so injection of ZnO NPs produced structural alterations in the seminiferous epithelium and sperm abnormalities in male rats.[15]

MATERIALS AND METHODS

Adult Male Sprague-Dawley albino rats, age about 2.5–3 months, average bodyweight 200–225 g. They were obtained from the National Center for Drug Control and Research/ministry of health, then transferred to the animal house of the college of science, Mustansyriah University. All animals were allowed to acclimatize to the laboratory conditions for 7 days before starting the study. They were kept in clean separated plastic cages with metal network cover under climate-controlled condition of the animal house with 22–25 temperature, 60% humidity, 12 h light, and darkness period and allowed free access to food *ad libitum* and water. This study was conducted after obtaining the approval from the ethical consideration to deal with experimental animals that is the Ethical Committee of the College of Science, Mustansiriyah University and all ethical behavior with the laboratory animals was taken care and has priority in our work in accordance with the globally adopted guidelines of the animal care and experiments.

Preparation of ZnO NPs Solution

ZnO NPs used in this study was obtained from sky spring nanomaterials, they were in white to light yellow colored powder with 99.8% purity, particle size was 10–30 nm in diameter. The stock suspension was prepared by dissolving 1 g of powder ZnO in 10 ml of distilled water and then mixed by vortex for 10 min to prevent agglomeration, then distributed in to following groups:

Group of 60 mg/kg of ZnO NPs (high dose) 120 μl of stock +880 μl of distal water.
Group of 30 mg/kg of ZnO NPs (low dose) 60 μl of stock +940 μl of distal water.

Experimental Design

To study the effect of ZnO NPs, animals were divided into nine groups with six rats each as follows:

- Groups 1, 2, and 3 (control group), respectively, received intraperitoneal injection of distilled water for (7, 14, 28) days.
- Groups 4, 5, and 6 (the experimental groups); rats, respectively, received intraperitoneal dose (60 mg/kg) of ZnO NPs for 7, 14, and 28 days.
- Groups 7, 8, and 9: (The experimental groups); rats, respectively, received intraperitoneal dose (30 mg/kg) of ZnO NPs for 7, 14, and 28 days.

Measurement of the Levels of Hormones Concentration

It was represented by the enzyme immunoassay tests (TOSOH) for the quantitative determination of concentrations of thyroid gland hormones T3 according to Braverman *et al*.,[16] T4 according to Ormston *et al*.,[17] and thyroid-stimulating hormone (TSH) according to Fisher.[18] Furthermore, the reproductive hormone testosterone was measured according to Sigberg.[19]

Collection of Blood Samples

The end of each experiment animals were completely anesthetized by diethyl ether for several minutes, and blood samples were obtained by heart puncture collected in to non-heparinized tubes used in biochemical examination, 4 ml of blood collected from each rat was used to obtain sera (0.5–1.0) ml separated by centrifugation 3000 rpm for 5 min, then they were kept in −20°C until analysis for the measurement of the level of TSH, measurement of the level of T3, measurement of the level of T4, and measurement of the level of testosterone.

RESULTS

Thyroid Hormone Function

Statistical analysis for the effect of ZnO NPs on T3, T4, and TSH serum levels is shown in Figures 1-3. T4 (μg/dl) serum level displayed high significant decrease ($P < 0.01$) in both treated groups (30, 60) mg/kg (3.10 ± 0.01), (2.90 ± 0.02) (μg dl), respectively, at day 7 compared to control groups (3.34 ± 0.01) (μg/dl), at day 14 also showed high significant decrease ($P < 0.01$) at the same concentrations (2.71 ± 0.01), (2.21 ± 0.02) (μg/dl) when compared with control group (3.35 ± 0.01) (μg/dl), in addition to 28 period of time exposing to ZnO NPs observed high significant decrease ($P < 0.01$) of the level of T4 in different concentration (30, 60) mg/kg (1.91 ± 0.02), (1.66 ± 0.01) (μg/dl) compared with control groups (3.36 ± 0.01) (μg/dl), as shown in Figure 1.

Statistical analysis of the level of T3 (ng/ml) showed high significant decrease ($P < 0.01$) in both treated groups (30, 60) mg/kg (0.685 ± 0.007), (0.600 ± 0.010) (ng/ml), respectively, exposed to ZnO NPs for 7 days compared to the control groups (0.786 ± 0.008) (ng/ml), also there was high significant decrease ($P < 0.01$) in the level of T3 at both concentrations (30, 60) mg/kg at 14 days (0.581 ± 0.01), (0.438 ± 0.019) (ng/ml) in comparison to control groups (0.786 ± 0.008), and at 28 days (0.440 ± 0.01), (0.335 ± 0.01) (ng/ml) in comparison to control groups (0.795 ± 0.009) (ng/ml), as shown in Figure 2.

While serum level of TSH (μlU/ml) showed high significant decrease ($P < 0.01$) at different treatment durations exposing to ZnO NPs in both treated groups (30 and 60) mg/kg (0.186 ± 0.007), (0.190 ± 0.010) (ng/ml), respectively, for 7 days compared to the control groups (175 ± 0.008) (ng/ml), also there was high significant decrease ($P < 0.01$) in the level of (TSH) at both concentrations (30 and 60) mg/kg in 14 days (0.188 ± 0.01), (0.195 ± 0.019) (ng/ml), respectively, in comparison to control group (0.175 ± 0.008), and at 28 days (0.191 ± 0.01), (0.198 ± 0.01) (ng ml) in comparison to control group (0.174 ± 0.009) (ng/ml), as shown in Figure 3.

Testosterone

Statistical analysis of the present study of ZnO NPs on testis function is observed in Figure 4 there was a high significant decrease ($P < 0.01$) in testosterone level at 30 and 60 mg/kg for 7 days (160.83 ± 1.16), (149.00 ± 1.29) Ng/dl, respectively, compared to control group (184.67 ± 1.05) Ng/dl. Testosterone level exhibited significant decrease ($P < 0.01$) at day14

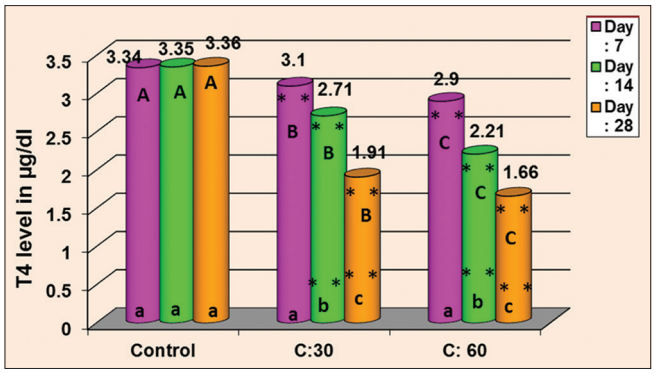

Figure 1: Effect of different concentrations of ZnO NPs (30 and 60) mg/kg on T4 levels of rats with different periods of time (7, 14, and 28) days in comparison with control groups and between treated groups themselves. **High significant decrease (≤0.01). [A,B,C]Represents the significant difference between groups with days as a fixed factor and concentrations as a variable factor. [a,b,c]Represents the significant difference between groups with concentrations as a fixed factor and days as a variable factor

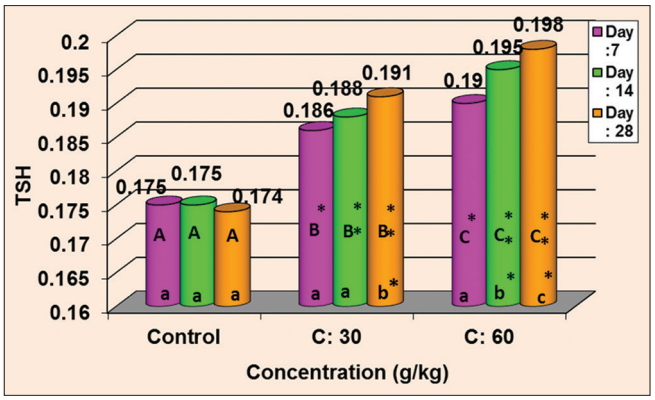

Figure 3: Effect of different concentrations of zinc oxide nanoparticles (30 and 60) mg/kg on thyroid-stimulating hormone levels of rats with different periods of time (7, 14, and 28) days in comparison with control groups and between treated groups themselves. **High significant decrease (≤0.01). [A,B,C]Represents the significant difference between groups with days as a fixed factor and concentrations as a variable factor. [a,b,c]Represents the significant difference between groups with concentrations as a fixed factor and days as a variable factor

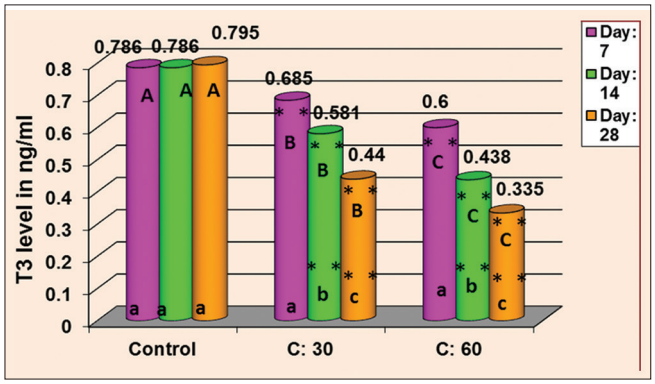

Figure 2: Effect of different concentrations of zinc oxide nanoparticles (30 and 60) mg/kg on T3 levels of rats with different periods of time (7, 14, and 28) days in comparison with control groups and between treated groups themselves. **High significant decrease (≤0.01). [A,B,C]Represents the significant difference between groups with days as a fixed factor and concentrations as a variable factor. [a,b,c]Represents the significant difference between groups with concentrations as a fixed factor and days as a variable factor

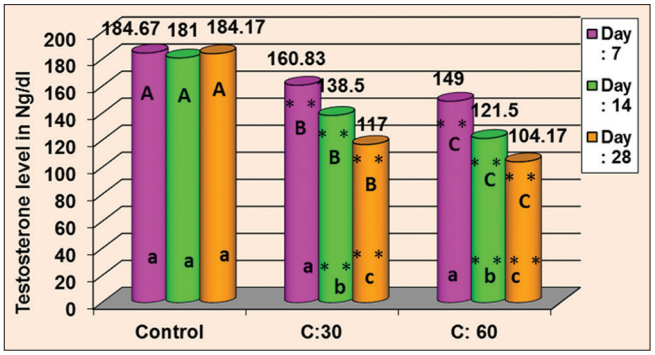

Figure 4: Effect of different concentrations of zinc oxide nanoparticles (30 and 60) mg/kg on testosterone levels of rats with different periods of time (7, 14, and 28) days in comparison with control groups and between treated groups themselves. **High significant decrease (≤0.01). [A,B,C]Represents the significant difference between groups with days as a fixed factor and concentrations as a variable factor. [a,b,c]Represents the significant difference between groups with concentrations as a fixed factor and days as a variable factor

when treated with ZnO NPs (30 and 60) mg/kg (138.50 ± 0.99), (121.50 ± 0.99) Ng/dl when compared with control groups (181.00 ± 1.31) Ng/dl. There was significant decrease ($P < 0.01$) showed in testosterone level at a period of 28 days treatment (30 and 60) mg/kg (117.00 ± 1.52), (104.17 ± 1.42) Ng/dl in comparison to control groups (184.17 ± 1.24) Ng/dl.

DISCUSSION

Results of the present study are in agreement with previous study of ZnO NPs by Cheric and Rafieirad[20] showed that injecting ZnO NPs with three different doses (1.25, 2.5, and 5) mg/kg to 42 male rats divided into 7 groups, the blood samples of experimental with different doses were taken on the 1st, 3rd, 4th, and 15th days after receiving Nano ZnO intraperitoneally (ip) to measure the amount of T3 and T4 hormone levels, results observed that acute injection of Nano ZnO reduced the amount of thyroid hormone T3 and T4, the effect occurred in different doses, but is more significant in the medium and long term, even in small amounts can cause negative effects on the activity of the thyroid gland and disrupt creation of thyroid hormones. Another study by Espanani *et al*.[21] who treated 48 male rats by ZnO NPs ip (5, 10, 20, and 40 mg/kg), after a 21 days period results showed significant increase in TSH hormone level in animals that were treated by high dose of ZnO NP, but those who treated by 5, 10, and 20 mg/kg do not show this effect. Dean *et al*.[22] Found a reduction in thyroid function T3, T4 when he fed chicken with Zn in diet at 73 ppm or 5280 ppm for 1–2 weeks, have demanded that high Zn intake change the production and secretion of thyroid hormones, they suggested that decrease in thyroid hormone function was due to reduced effects of thyroid gland might be induced by the reduced regulatory effect of pituitary gland on thyroid gland, and decreased circulating thyroid hormones

may be indicative of hypothyroidism due to Zinc toxicity. Lu *et al*.[23] Stated that Zn decrease the binding of T3 to its receptor in several preparations *in vitro*.

Kaya *et al*.[24] Conducted the experiment using 130 Hisex Brown laying hens from 56 weeks to 68 weeks of age, then they were divided into five zinc treatment groups (0, 25, 50, 100, and 200) mg/zinc/kg diet respectively, the values of 100 and 200 mg/Zn/kg decreased plasma level of T4 compared to control, while plasma level of T3 was reduced by 100 mg/Zn/kg compared to groups fed less Zn, these data might suggest that a high intake of Zn changes production or secretion of the thyroid hormone.[25] Used 12 lambs and 12 goats that were divided into two equal groups as control and Zn groups in separate experiments, both species of animals in the Zn groups were fed a basal ration supplemented with zinc sulfate adjusted to 250 mg Zn/kg diet showed significant decrease in hormone level T3 and T4.[26] Randomly distributed 120 male rabbits into four groups, the control groups were fed on a basal diet with zinc-free premix, while experimental groups received the basal diet supplemented, Group 1 with 60 mg/kg nano ZnO/kg diet, Group 2 60/kg mg nano ZnO/kg diet, and Group 3 30/kg mg nano ZnO/kg diet, respectively, results observed that rabbits showed no significant changes among the treated groups in respect to serum TSH concentration. In contrast, Baweja *et al*.[27] demonstrated that the levels of TSH were raised after 8 weeks of zinc supplementation in female Wistar rats. Another study that disagree with present report by Shirband *et al*.[28] that administered iron oxide NPs with three different dose (20 μg/kg, 50 μg/kg, and 150 μg/kg) for 15 days to male rats, results showed significant increase in T4 level in groups receiving a dose 50 μg/kg and caused significant decrease in TSH in the group receiving a dose 50 μg/kg and 150 μg/kg. It is likely that NP effects can be applied through the inhibition of endocrine pituitary axis hypothalamus which affects the hypothalamus, and probably due to decreased TSH levels. NPs affect hypothalamic pituitary thyroid axis and therefore affect the level of thyroid hormones that were demonstrated in this study. In a study by[29] used 32 adult male mice treated daily for 35 days: 5, 50, and 300 mg/kg ZnO NPs, respectively, while control group received distilled water orally for 35 consecutive days, results observed significantly changed in 50 and 300 mg/kg ZnO NPs treated mice in epididymal sperm parameters including sperm number, motility, and percentage of abnormality, while significant decrease in seminiferous tubule diameter, seminiferous epithelium height, and maturation arrest was observed at 50 and 300 mg/kg ZnO NPs, this study established that ZNP has cytotoxic actions on testicular germ cells in a dose-dependent manner, NPs might also affect Sertoli cell functions.[30] Who treated 40 female Wistar rats with zirconium oxide NPs with a dose of 100, 200, and 400 ppm injected intraperitoneally showed a significant decrease in level of testosterone at high doses. Reports of a study by Mohammadi *et al*.[31] injected Titanium dioxide TiO NPs intraperitoneally to Wistar rats weighing 150–250 g, 1 ml TiO2 NPs in doses (30 and 50) mg/kg, injection repeated every other day, results showed decrease in testosterone level, could be caused by adverse effects of NPs in the Leydig cells, as Leydig cells are testosterone production factories. Another study by Fuse *et al*.[32] showed that zinc NPs disorder leads to atrophy to the seminiferous tubules and impaired spermatogenesis in male rats. In a study by Shirvani *et al*.[33] was intraperitoneal injected of different doses (25, 50, and 100 mg/kg) of ZnO NPs (25 nm) to male Wistar rats showed decrease but no significant in level of testosterone, synthesis of testosterone is inhibited as a result of responding to the inflammation which is caused by ZnO NPs.[34] Treated rats with ZnO NPs with dose (5, 10, 20, and 40 mg/kg) results revealed significant increase in level of testosterone in blood serum of rats at high dose of ZnO NPs (40 mg/kg), results assured exposing to ZnO NPs damaged to public health and reduced fertility potential. ZnO NPs are related to (ROS), which results in an increase in DNA double-strand breakage and a decrease in sperm motility.[33] Kolesarova *et al*.[35]reported that metal NPs induce changes in reproductive organs, histology of laboratory animals and cause disruption in reproductive cells production and hormones. Thyroid hormone deficiency affects all tissues of the body including multiple endocrine changes that alter growth hormone, corticotrophin, gonadal function, and glucocorticoids, as primary hypothyroidism is associated with hypogonadotropic hypogonadism, so hypothyroidism decrease free testosterone concentration as thyroid hormone affects in sex hormone-binding globulin (SHBG).[36] Alterations in gonadal steroid genesis and pituitary functions have been stated in hypothyroid males, hypothyroidism was found to be associated with an increase in level of total cholesterol and reduction in the levels of testosterone and progesterone without any alteration in the levels of gonadotropins and estradiol, the decline in level of testosterone could be explained by reduction in serum triiodothyronine, a higher rate of alteration of testosterone to estradiol or the further decline in the rate of alteration of progesterone to testosterone.[37] Increasing in TSH level that was caused by ZnO NPs decreased gonadotropin-releasing hormone secretion by negative feedback inhibition that led to LH and FSH reduction, while inhibin hormone that is usually released by Sertoli cells can affect FSH hormone level.[21] Considering the results of the above mentioned studies and the alterations in results from the present study, these outcomes could be related to the dosage of ZnO NPs used, animal species diversity, route of administration of NPs and different durations of exposing to NPs, NPs showed cytotoxic effect on testosterone and thyroid hormone levels in dose and time-dependent manner.

CONCLUSIONS

The results of this study showed that the size, doses, route of administration, and time depended can be a factor that effects thyroid hormone level and testosterone, the decrement in T3 andT4 can be caused by dose and duration of ZnO NPs, decrease in levels of thyroid hormones due to toxic effect of ZnO NPs that effects on the function of thyroid gland or on release of TSH. NPs affects the reproductive system of male by complex and varied mechanisms; results indicate that thyroid hormone deficiency has effects on testosterone level, as hypothyroidism decrease free testosterone concentration, also thyroid hormone has effects on SHBG.

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Cihan University-Erbil Scientific Journal (CUESJ)

Research Article

Investigating the Inhibitory Effect of Silver Nanoparticles against Some Species of *Candida* and Pathogenic Bacteria

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ABSTRACT

Silver nanoparticles synthesized from aqueous extract of mushroom *Pleurotus ostreatus* exhibited inhibitory effect at the concentration of 12.5, 25, 50, and 100 mg/ml against some pathogenic bacteria and fungi such as *Candida albicans, Candida guilliermondii, Candida krusei, Candida zeylanoides, Geotrichum klebahnii, Staphylococcus aureus, Pseudomonas aeruginosa,* and *Escherichia coli.* The maximum inhibition zone was observed against *C. zeylanoides* at the concentration of 100 mg/ml was 24.5 mm, while the minimum inhibition zone was observed against *Geotrichum* at the concentration of 25 mg/ml was 8 mm and the concentration of 12.5 mg/ml was not effective against some species.

Keywords: Pathogenic *Candida*, *Pleurotus ostreatus,* silver nanoparticles

INTRODUCTION

A new dimension of the metal microbial interaction has been explored for the synthesis of metal nanoparticles such as gold, silver, cadmium, zirconia, and silica titanium.[1] Silver compounds have been used to treat burns, wounds, and infections as well as in preventing bacterial colonization of prostheses and catheters.[2] Various salts of silver and their derivatives are used as antimicrobial agents. Nanosized silver particles exhibit antimicrobial properties. Nanoparticles of silver have been studied as a medium for antibiotic delivery, and to synthesize composites for use as disinfecting filters and coating materials.[3] The nanoparticles were important due to their emission properties. These nanoparticles are used for wide range of application.[4,5] Antibacterial activity is related to compounds that locally kill bacteria or slow down their growth, without being in general toxic to surrounding tissue.[6] The high bactericidal activity is certainly due to the silver cations released from Ag nanoparticles (AgNPs) that act as reservoirs for the Ag+ bactericidal agent.[7] Severe fungal infections have significantly contributed to the increasing morbidity and mortality,[8] immunocompromised patients who need intensive treatment including broad-spectrum antibiotic therapy,[9,10] and *Candida* spp. represent one of the most common pathogens which are responsible for fungal infections often causing hospital-acquired sepsis with an associated mortality rate of up to 40%.[11] Currently, the majority of yeast species are resistant the available antifungal therapy[12,13] such as on polyenes (amphotericin B), triazoles (fluconazole, itraconazole, voriconazole, and posaconazole) or echinocandins (caspofungin, micafungin, and anidulafungin) which exhibit their toxicity, adverse effects, and drug interaction.[14,15]

The objective of this work is to evaluate the effect of a biosynthesized AgNPs product against some *Candida* spp. and bacteria species to determine how AgNPs interact with the growth of microbial cells.

MATERIALS AND METHODS

The mushroom *Pleurotus ostreatus* was obtained from fruit body grown on peach tree in Salah-Alddin Province.

Preparation of Hot Aqueous Extracts of Mushroom

The oven dried mushroom was blended; the obtained powder was soaked in distilled water at a ratio of 1:10 (w/v) and boiled with agitation at 60 ± 2°C for 30 min. The boiled mushroom powder was then left covered for 30 min. Residues were then removed by filtration through gauze and further centrifugation (10,000 rpm, 30 min, and

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4°C). Supernatants were then collected and filtered through Whatman No. 1 filter paper. After that, freeze dried extract powders were obtained using freeze dryer and stored at 4 ± 2°C.[16]

Biosynthesized AgNPs from *P. ostreatus*

Silver nitrate (1 × 10^{-3}) AgNO3 stock solution was prepared in sterile deionised triple - distilled water and the subsequent dilutions were made from this stock solution. The bulk amount of (10) mg/ml of aqueous extract solution of is prepared with sterile distilled water and filtered through syringe filter (0.2) μm. Based on the result of a preliminary trial, 2–7 ml of 10 mg/ml of an aqueous extract of *P. ostreatus* (P2) were filled with sterile distilled water to a total of 10 ml.

After that, the solution is added to 5 ml of (1 × 10^{-3}) M aqueous AgNO3 solution and kept at room temperature and exposed under ultraviolet (UV) (365) nm (long UV). After 24 h incubation, the light yellow color of the mixture solution turned to dark yellow indicating the formation of AgNPs.[17]

Candida Isolates

All *Candida* isolates *Candida albicans, Candida guilliermondii, Candida krusei, Candida zeylanoides,* and *Geotrichum klebahnii* were procured from AL-Yarmouk Teaching Hospital. Mycological identification was carried out for all samples by commercial carbohydrate assimilation systems such as the API 20 C test kit.[18] All yeast isolates were inoculated in a primary isolation medium such as Sabouraud Dextrose Agar (SDA) medium for 2–3 days at 37°C.

Antimicrobial Activity of AgNPs (Well Diffusion Method)

The synthesized AgNP was tested for antimicrobial activity by agar well diffusion method against pathogenic microbes for all the previously mentioned bacteria and yeast species. The pure cultures of bacteria and yeast were subcultured on nutrient and SDA subsequently. Each strain was swapped homogeneously onto the individual plates using sterile cotton swabs. Wells of 10 mm diameter were done. The concentration of AgNPs was poured on each well. After 24 h of incubation the diameter of inhibition zone was measured. Three replicates of experiments were carried out.[19]

RESULTS

The formation of AgNPs was confirmed by the UV-visible spectrophotometry, which showed a strong peak within the range of 200–500 nm, "Figure 1."

Intensity of Particle Size Distribution Analysis

To know the size of synthesized AgNPs, size distribution analysis was performed using light scattering in aqueous solution. The results showed that the size of the particles ranged from 16 to 104 nm. The average size of AgNPs was determined to be 49 ± 16 nm as in "Figures 2 and 3."

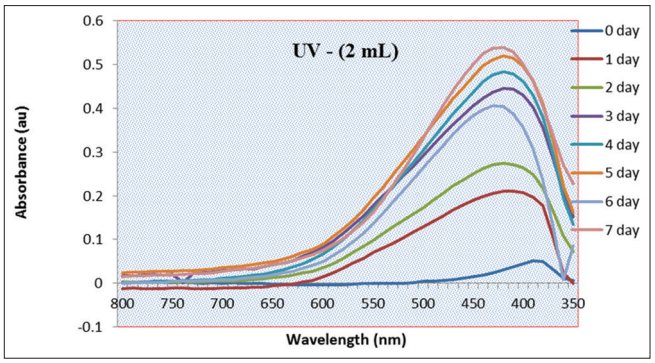

Figure 1: Ultraviolet-visible absorption spectra of silver nanoparticles after bioreduction by *Pleurotus ostreatus* mushroom aqueous extract

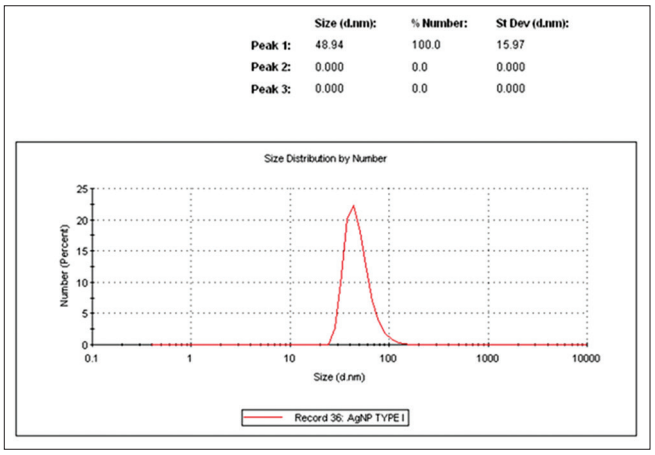

Figure 2: Size of silver nanoparticles

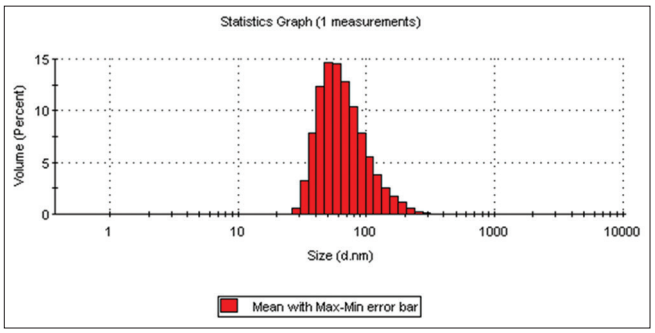

Figure 3: Intensity of particle size distribution of *Pleurotus ostreatus* dried mushroom aqueous extract

DISCUSSION

Antimicrobial mechanisms of nanomaterials are not fully understood, but it is proposed that when they come into contact with cells, they provoke the production of reactive oxygen specie, cell membrane disruption, mitochondrial damage, and DNA damage.[20]

Antifungal activity by AgNPs has been proved against different *Candida* species, including *C. albicans*, *since microbial cells* were inhibited using AgNPs.[21] Furthermore, it was reported that AgNPs damage the structure of the cell membrane in *C. albicans* producing "holes" on the surface of the cells and thus inhibiting the budding process.[22]

Table 1: The inhibitory effect of AgNPs (mm) against *Candida* and bacterial species

***Candida* spp.**	**Inhibition zone (mm)**			
	12.5 mg/ml	**25 mg/ml**	**50 mg/ml**	**100 mg/ml**
Candida albicans	---	10	12	16
Candida guillermondii	---	10	15.5	20
Candida krusie	---	14.5	15.5	19
Geotrichum ktebahnii	---	8	14	20
Candida zeylanoides	15	18.5	21	24.5
Staphylococcus aureus	10	11	12	16
Pseudomonas aeroginosa	---	12	13	14
Escherichia coli	10	12	13	15

AgNPs: Silver nanoparticles

Table 2: Statistical analysis of the inhibitory effect of AgNPs (mm) on *Candida* and bacterial isolates

Microbial species	**Control**	**AgNPs (mg/ml) s**			
		12.5	**25**	**50**	**100**
Candida albicans	$<0.001^{d}$	0 ± 0.0^{d}	10 ± 0.57^{c}	12 ± 0.58^{b}	16 ± 0.58^{a}
Candida guillermondii	$<0.001^{d}$	0 ± 0.0^{d}	15 ± 0.58^{c}	15.6 ± 0.33^{b}	20 ± 0.57^{a}
Candida krusie	$<0.001^{d}$	0 ± 0.0^{d}	14.5 ± 0.28^{c}	15.6 ± 0.16^{b}	19 ± 0.57^{a}
Geotrichumktebahnii	$<0.001^{d}$	0 ± 0.0^{d}	8 ± 0.57^{c}	14 ± 0.58^{b}	20 ± 0.58^{a}
Candida zeylanoides	$<0.001^{e}$	15 ± 0.57^{d}	18.5 ± 0.28^{c}	21 ± 0.57^{b}	24.5 ± 0.28^{a}
Staphylococcus aureus	$<0.001^{d}$	10 ± 0.58^{c}	11 ± 0.57^{bc}	12 ± 0.57^{b}	16 ± 0.58^{a}
Pseudomonas aeroginosa	$<0.001^{c}$	0 ± 0.0^{c}	12 ± 0.58^{b}	13 ± 0.58^{ba}	14 ± 0.57^{a}
Escherichia coli	$<0.001^{d}$	10 ± 0.57^{c}	12 ± 0.58^{b}	13 ± 0.57^{b}	15 ± 0.58^{a}

The findings were described in the table represent the average of three replicates±standard error. Small letters (a, b, c, d, e, ba, and ac) indicate to comparison between means in column, similar letters are non-significantly differences between means at ($P\leq0.05$), using (LSD test). AgNPs: Silver nanoparticles

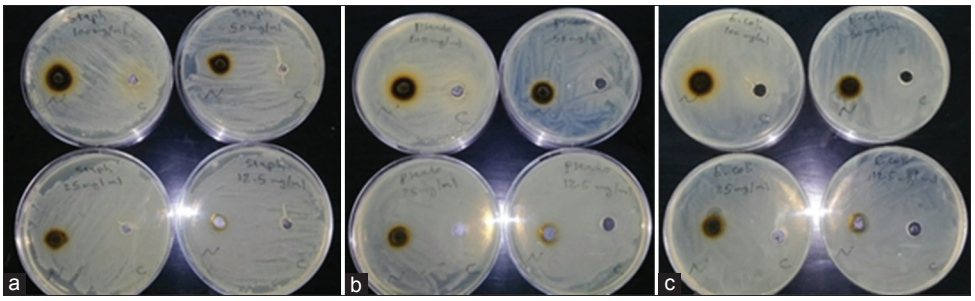

Figure 4: Inhibition zone of silver nanoparticles against several isolates of bacteria; (a) *Staphylococcus aureus*, (b) *Pseudomonas aeroginosa* (c), *Escherichia coli*

The antifungal properties of AgNPs against *C. albicans* have been demonstrated in some other studies, although reported minimum inhibitory concentration values are different from the ones we found in this work.[21-24] Such differences could be due to the nature of the particles used, the difference in size being particularly important. It is known that size and shape of metallic nanoparticles influence their chemical, optical, and thermal properties.[25]

The effectiveness of AgNPs against bacteria is clearly demonstrated, and in fact, AgNPs were shown to be effective against *E. coli*, with cells showing formation of "pits" in the cell wall. The AgNPs were found to accumulate in the bacterial membrane and some of them were reported to successfully penetrate into the cells.[26] Similar results were found in *E. coli* and *Vibrio cholera*; it was established that AgNPs provoked changes mainly in the cell membrane morphology, producing a significant increase in their permeability, thus affecting the proper transport through the plasma membrane, and resulting eventually in cell death. They also reported that silver NPs with small diameters penetrated into the cells.[27] In our study, Ag NPs were found surrounding *C. albicans* cells, similar to the results found in bacteria.[27-29]

In this study, the aqueous extracts of mushroom (*P. ostreatus*) gave showed (ve−) results against all microbial species when used as a control [Figures 4 and 5], while the biosynthesized AgNPs gave showed (ve+) result as an inhibition zones for different microbial species [Figures 4 and 5, Tables 1,2].

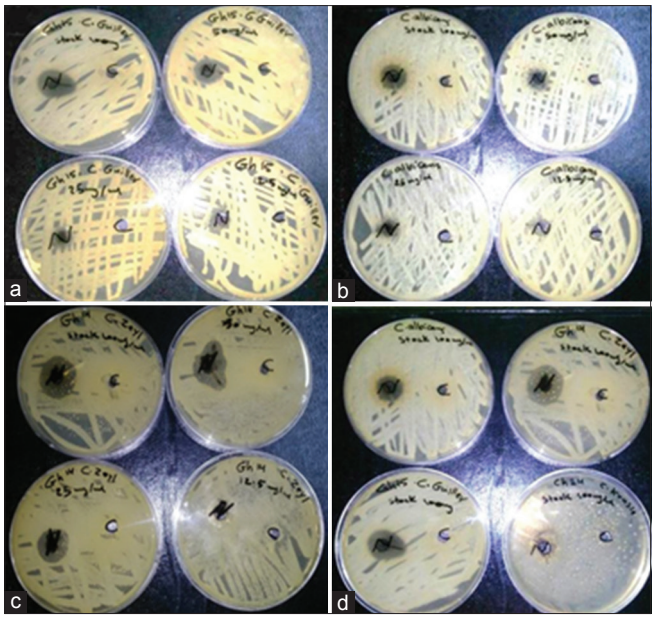

Figure 5: Inhibition zone of silver nanoparticles (AgNPs) (N) against several isolates of *Candida*; (a) *Candida guillermondii*; (b), *Candida albicans*; (c), *Candida zeylanoides*; (d), *Candida* spp. in concentration of 100 mg/ml of Ag NPs the way of measuring of diameter of inhibition zone. The aqueous solution of *Pleurotu sostreatus* (C)

However, despite the clear antimicrobial properties of AgNPs, their potential use in the clinic should be carefully evaluated since there is a lack of basic knowledge on the potentially different antimicrobial properties of AgNPs which may vary depending on many factors, including the method of synthesis, size, shape, functionalizing agent, and application method and also their interaction in more complex systems such as plants, animals, and humans.

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Research Article

Nutritional Characteristics of Pregnant Women and its Relation with Anemia during Pregnancy in a Sample of Kurdish Women/Iraq

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ABSTRACT

Anemia in pregnancy is a major public health problem, especially in developing countries. This study aimed to assess the nutritional characteristics of pregnant women and find out its relationship with anemia during pregnancy. A descriptive, cross-sectional study was conducted on 600 pregnant women who attended four primary health-care centers which randomly selected according to the geographical area. A specially designed questionnaire was prepared by the researcher after extensive review of relevant literature. The severity of anemia is determined according to Alene and Dohe. Estimation and calculation of dietary characteristics was done according to food frequency questionnaire, frequencies and percentage, mean and standard deviations, and Chi-square test of association and regression analysis. There was a highly statistically significant association between anemia with eating vegetables and chicken, and a high significant relation of anemia with eating beef and eating nuts. Furthermore, there was a highly significant association between severity of anemia with eating nuts, and a significant association with eating vegetables, while there was no significant association with other variables. Logistic regression analysis revealed that eating less than normal of vegetables, beef, and nuts were indicated risks of anemia. Eating less than normal of vegetables, beef, and nuts were indicated risks of anemia during pregnancy.

Keywords: Anemia, diet, nutrition, pregnancy

INTRODUCTION

Anemia in pregnancy is a major public health problem, especially in developing countries. It affects 41.8% of pregnant women globally.[1] Anemia in pregnancy is defined by the World Health Organization (WHO) as a hemoglobin (Hb) concentration below 11 g/dL.[2]

Nutritional anemia is the most common type of anemia worldwide and mainly includes iron, folic acid, vitamin B_{12}, and Vitamin C deficiencies. Iron deficiency contributes to half of the burden of anemia globally. Iron deficiency affects 1.3–2.2 billion persons out of that 50 % are women of reproductive age. In Ethiopia, nearly 17% of women with age of 15–49 years are anemic of these 22% are pregnant women.[3]

Anemia in pregnant women has severe consequences on health, social, and economic development. Anemic pregnant women will be at risk of low physical activity, increased maternal morbidity and mortality, especially those with severe anemia. In addition, both pregnant women and their neonates encounter negative consequences including fetal anemia, low birth weight (LBW), preterm delivery, intrauterine growth restriction, and perinatal mortality.[1]

A large number of women experience micronutrient deficiencies (of iron and Vitamin A, for instance); almost half of all pregnant women in the world are thought to have anemia and 9.8 million pregnant women have night blindness. An estimated 19.1 million pregnant women (the highest proportions in Africa and South-Eastern Asia) have low serum retinol concentrations. Maternal deficiencies in micronutrients may lower infant birth weight and jeopardize development and survival: Maternal iodine deficiency is associated with congenital malformations, and mental retardation in children and a link between Vitamin B12 deficiency and an increased risk of diabetes has been described in India. Insufficient intake of specific fatty acids, such as docosahexaenoic acid, may also impede children's development.[4]

During pregnancy, a woman needs good nutritional status for a healthy outcome. Women who have a poor nutritional

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status at conception are at higher risk of disease and death; their health depends greatly on the availability of food, and they may be unable to cope with their increased nutrient needs during pregnancy in situations of food insecurity. Maternal nutrition is a fundamental determinant of fetal growth, birth weight, and infant morbidity; poor nutrition often leads to long-term, irreversible, and detrimental consequences to the fetus.[4]

Studies have shown that the adverse consequences of maternal anemia may affect not only the neonate and infant but also increase the risk of non-communicable diseases when the child grows into an adult and the risk of LBW in the next generation.[5] Low maternal Hb levels are associated with increased risk of preterm delivery, LBW babies, Apgar score <5 at 1 min and intrauterine fetal demise.[6]

According to the results of two previous studies in Erbil city regarding anemia during pregnancy, the prevalence of anemia was 55.5%, and 70% of participants had risk factors.[7,8] According to the knowledge of the researcher, no study focuses on dietary characteristics of anemic women. Because the anemia during pregnancy is still a major public health problem and a high prevalence in Kurdish women, therefore the researcher interested to study it concerning diet of them. Therefore, this study aimed to assess the nutritional characteristics of pregnant women and find out its relationship with anemia during pregnancy.

METHODS

A descriptive, cross-sectional study was conducted to assess the nutritional characteristics of pregnant women in Primary Health Care Centers (PHCC) of Erbil city, Kurdistan Region, Iraq. The study was conducted during the period October 1, 2015–November 13, 2016. The period included data collection, analysis, and interpretation. The study was conducted among pregnant women who attended antenatal care in PHCC in Erbil city. During time of data collection, 21 primary health centers were in Erbil city which 19 of them were provided with maternal and child health care services. Four PHCC were randomly selected according to the geographic area from North, South, East, and West. A purposive sampling of 600 pregnant women was included in the study. The sample size was estimated using the general formula for targeted population size, allowed error 5%, prevalence (ratio of the studied phenomenon in the population in similar study) and using the 95% confidence interval. The sample size of each PHCC was according to their ratio in the targeted population. The sample size from Kurdistan PHCC was 117, Brayati was 168, Mahamad Bajalan was 117, and Malafande was 198. Mothers who had the following criteria were included in the study: Pregnant women who were in 1st, 2nd, and 3rd trimester of pregnancy and woman who accept to participate in the study. Pregnant women with history of chronic hypertension, diabetes, thyroid, cardiac diseases, and systematic lupus erythematosis, woman's with hemoglobinopathies such as thalassemia and woman's who have early and late vaginal bleeding or antepartum hemorrhage (abruption placenta and placenta previa) and hemorrhoid were excluded from the study.

Before data collection, the official permission was obtained from College of Nursing, Erbil General Directorate of Health, and PHCC for carrying out the study in Erbil City. Face to face interview method and reviewing antenatal card were used for data collection after taking permission and explanation the objectives of the study to the mother's. Each interview session took approximately 15–20 min. Blood Hb level was measured to assess severity of anemia. The severity of anemia is determined according to Alene and Dohe (2014) as the following: Mild (Hb level between 10.0 and 11 g/dl), moderate (Hb level between 7.0 and 9.9 g/dl), and sever (Hb level <7 g/dl).

A specially designed questionnaire was prepared by the researcher after extensive review of relevant literature, which consists of three parts: Socio-demographic characteristic obstetrical characteristics, dietary characteristic of pregnant women which included how often did the mother eat the followings: Fruit, vegetables, chicken, fish and seafood, beef, other meat, nuts, beans, dairy, eggs, grains, sweets, caffeinated soft drinks, coffee and tea, and what was the usual serving size for each of them. Estimation and calculation of dietary characteristics was according to Food Frequency Questionnaire (FFQ).

The validity of the study questionnaire was presented through panel of 14 experts of different specialties related to the field of the present study. They recommended some modifications and suggestions regarding some questions and all responses were taken into consideration in the final draft of the questionnaire format. A pilot study was conducted on 20 mothers who attended antenatal care units. According the results of the pilot study, some items of the questionnaire were removed and other was added to the questionnaire form. The proposal of the study was approved by Ethical Committee from the College of Nursing and General directorate of PHCC. Informed consent was taken from study participants. Data were entered into a computer using the Statistical Package for the Science Services (SPSS version 23) and following statistical procedures were applied: Frequencies and percentage, mean and standard deviations, and Chi-square test of association and regression analysis.

RESULTS

The highest percentage of 57.3% of the study sample aged between 25 and 36 years old. All participants of the study sample were living in urban areas. The highest percentages of 34.3% of the study sample were graduated from institute, college, and above. Regarding occupation, the highest percentages of 78.7% of the study samples were unskilled workers. Regarding economic status level, the highest percentage of 49.7% of the study sample had low economic status [Table 1].

Table 2 shows that the highest percentage of 59.2% of the study sample regarding age at the first pregnancy was in age group 13–23 years; regarding gravidity, the highest percentage of 64.3% of the study sample was multigravida; concerning parity, the highest percentage of 48.3% of the study sample was nulliparous, and the highest percentage of 71.5% of the study sample had no abortion.

Table 3 shows that the highest percentage of 53.2% of the study sample was in second trimester. The highest percentage of 92.3% of the study sample took iron and folic acid supplementation; the highest percentage of 87% of the study

Table 1: Socio-demographic characteristics of the study sample (n=600)

Variables	F	%
Age group/years		
13–24	208	34.7
25–36	344	57.3
37–48	48	8
Wife education		
Illiterate	96	16
Read and write only	24	4
Primary school	106	17.7
Intermediate school	85	14.2
Secondary school	83	13.8
Institute, college, and above	206	34.3
Wife occupation		
High professional job	12	2
Lower professional job	116	19.3
Unskilled workers	472	78.7
Economic status level		
Low economic status <90	298	49.7
Middle economic status 90–120	177	29.5
High economic status 121–150	125	20.8

Table 2: Obstetrical characteristics of the study sample (n=600)

Variables	F	%
Age at first pregnancy		
13–23	355	59.2
24–34	229	38.2
35–45	16	2.7
Gravidity		
Primigravida	214	35.7
Multigravida	386	64.3
Parity		
Nulliparous	290	48.3
Primiparous	142	23.7
Multiparous	96	16
Grand multipara	72	12
Abortion		
None	429	71.5
1–3	171	28.5
Trimester		
First trimester	33	5.5
Second trimester	319	53.2
Third trimester	248	41.3
Iron, folic acid, Fero-folic supplementation		
Yes	554	92.3
No	46	7.7
Number of iron and folic acid tablet taking/month		
<30	522	87
≥30	32	5.3
None	46	7.7
Anemia before pregnancy		
Yes	13	2.2
No	587	97.8

Table 3: Prevalence and severity of anemia among the study sample

Prevalence	F	%
Anemic	277	46.2
Non-anemic	323	53.8
Total	600	100
Severity of anemia		
Moderate	91	32.9
Mild	186	67.1
Total	277	100

sample took <30 tabs/month, the highest percentage of 97.8% of the study sample did not have anemia before pregnancy.

Table 4 shows the prevalence and severity of anemia of the study sample. Prevalence of anemia during pregnancy was 46.2%, regarding the severity of anemia the highest percentage of 67.1% of the study sample had mild anemia, while the lowest percentage of 32.9% of the study sample had moderate anemia, and there were no cases for severe anemia.

Table 5 shows the dietary characteristics of the study sample for eating daily frequency, serving size, and daily amount. The highest mean for eating daily frequency was related to drinking coffee and tea 1.99, eating fruit 1.55, eating dairy 1.52, and 1.17 eating grains, while the lowest mean 0.24 eating fish and seafood, 0.31 caffeinated soft drinks, and 0.47 eating beef. Regarding serving size, the highest mean was 0.98, 0.96, 0.95, and 0.93, respectively, for drinking coffee and tea, eating fruit, eating eggs, and eating dairy, while the lowest mean was 0.50 and 0.51 for eating chicken and beef and fish and seafood, respectively. Regarding daily amount, drinking coffee and tea, eating fruit, dairy, and eggs had the highest mean (1.95. 1048, 1.41, and 0.84, respectively), while the lowest mean of daily amount was 0.12 for eating fish and seafood, 0.18 caffeinated soft drinks and 0.23 eating beef.

Table 6 shows that all of the study samples had dietary daily amounts less than normal regarding fish and seafood, beans, grains, and eggs, while fruits 92.8%, coffee and tea 83.8%, and nuts 81.5% had the highest normal dietary amounts. More than half of the study sample had normal dietary daily amounts of dairy 54.7% and beef 52.2%. Furthermore, it shows that there was a highly statistically significant association between anemia with eating vegetables and chicken ($P < 0.001$), and a highly significant relation of anemia with eating beef ($P < 0.01$), and eating nuts ($P < 0.05$). There was no significant association of anemia with other variables ($P > 0.05$) such as eating fish and seafood, beans, eggs, and grains.

Table 4: Dietary characteristics of the study sample (n=600)

Dietary characteristics	Eating daily frequency (mean)	Serving size (mean)	Daily amount (mean)
Fruit (apples, bananas, oranges, etc.)	1.55	0.96	1.488
Vegetables (carrots, mushrooms, potatoes, etc.)	0.92	0.57	0.524
Chicken (fried chicken, in soup, grilled chicken, etc.)	0.62	0.50	0.31
Fish and seafood (tuna, shrimp, crab, etc.)	0.24	0.51	0.122
Beef (steak, meatballs, etc.)	0.47	0.50	0.235
Nuts (almonds, cashews, walnuts, etc.)	0.86	0.63	0.541
Beans (tofu, chickpeas, chili, etc.)	0.82	0.56	0.459
Dairy (cheese, milk, yogurt, etc.)	1.52	0.93	1.413
Eggs (omelet, in salad, etc.)	0.89	0.95	0.845
Grains (bread, pasta, rice, etc.)	1.17	0.63	0.737
Sweets (candy, cookies, pie, etc.)	0.67	0.71	0.475
Caffeinated soft drinks (cola, diet cola, energy drinks, etc.)	0.31	0.59	0.182
Coffee and tea (hot coffee, iced coffee, black tea, etc.)	1.99	0.98	1.95

Table 5: Dietary daily amounts of the study sample (n=600)

Dietary daily amount	Less than normal		Normal	
	F	%	F	%
Fruit (apples, bananas, oranges, etc.)	43	7.2	557	92.8
Vegetables (carrots, mushrooms, potatoes, etc.)	504	84	96	16
Chicken (fried chicken, in soup, grilled chicken, etc.)	588	98	12	2
Fish and seafood (tuna, shrimp, crab, etc.)	600	100	0	0
Beef (steak, meatballs, etc.)	287	47.8	313	52.2
Nuts (almonds, cashews, walnuts, etc.)	111	18.5	489	81.5
Beans (tofu, chickpeas, chili, etc.)	600	100	0	0
Dairy (cheese, milk, yogurt, etc.)	272	45.3	328	54.7
Eggs (omelet, in salad, etc.)	600	100	0	0
Grains (bread, pasta, rice, etc.)	600	100	0	0
Sweets (candy, cookies, pie, etc.)	533	88.8	67	11.2
Caffeinated soft drinks (cola, diet cola, energy drinks, etc.)	582	97	18	3
Coffee and tea (hot coffee, iced coffee, black tea, etc.)	97	16.2	503	83.8

Table 7 shows that there was a highly significant association between the severity of anemia with eating nuts ($P < 0.01$), and a significant association with eating vegetables ($P < 0.05$), while there was no significant association with other variables ($P > 0.05$).

Logistic regression analysis revealed that eating less than normal of vegetables, beef, and nuts were indicated risks of anemia ($P < 0.05$ odds ratio [OR] = 0.543, 0.147, 0.611, 0.493, 0.689, 0.641, respectively). On the other hand, eating less than normal of chicken was indicated no risks of anemia ($P > 0.05$ OR = 3.062, 1.433, 1.039, respectively) [Table 8].

DISCUSSION

Prevalence of anemia in the present study was 46.2% which indicated severe public health problems, which was similar to the results of other studies done in Bali, Indonesia in 2002 which was 46.2%, in Northern Tanzania was 47.4% in 2011, and 48.2% in South-East Asia, and 44.2% in Eastern Mediterranean by WHO 2005, in South-Eastern Nigeria in 2007 was 40.4%, in Kakamega County, and Kenya was 40% in 2014. Prevalence of anemia in the present study was higher than the results of other studies done in Wolayita Sodo Town, Southern Ethiopia in 2015 was 39.94%, in Makkah, Saudi Arabia was 39% in 2012, in Southeast Ethiopia was 27.9% in 2014, in Europe was 25.1%, and in Americas 24.1% by WHO 2005, in Nablus, Palestine was 21.7% in 2007, in Mekelle town was 19.7% in 2014, in the lower North of Thailand was 17.5% in 2012, in Kerman, and Iran was 4.7% in 2010.

The results of a study done by Kefiyalew *et al.*[9] found that 55%, 32.5%, and 12.5 of the study sample had mild, moderate, and severe anemia, respectively, which was consistent with the results of the present study. Dim and Onah[2] reported that 90.7% had mild anemia, 9.3% had moderate anemia, and no cases of severe anemia were detected on their studies which were consistent with the results of the present study.

Women from lower socioeconomic groups are at higher risk of inadequate protein intake due to the associated costs. They are also more likely to choose less expensive processed foods which would put them at risk of small for gestational age babies.[10]

Results of the present study, there was a high significant ($P < 0.001$) association of anemia with eating vegetables, chicken, and beef, and a significant ($P < 0.05$) association with eating nuts, while there was no significant ($P > 0.05$) association between anemia and eating of fruits, dairy,

Table 6: Association of dietary daily amounts with anemia during pregnancy (n=600)

Variables	Anemic		Non-anemic		*P*-value Chi-square test
	F	%	F	%	
Fruit (apples, bananas, oranges, etc.)					0.962
Less than normal	20	46.5	23	53.5	
Normal	257	46.1	300	53.9	
Vegetables (carrots, mushrooms, Potatoes, etc.)					0.002
Less than normal	219	43.5	285	56.5	
Normal	58	60.4	38	39.6	
Chicken (fried chicken, in soup, grilled chicken, etc.)					0.001
Less than normal	277	47.1	311	52.9	
Normal	0	0.0	12	100.0	
Fish and seafood (tuna, shrimp, crab, etc.)					Constant
Less than normal	277	46.2	323	53.8	
Normal	0	0.0	0	0.0	
Beef (steak, meatballs, etc.)					0.004
Less than normal	115	40.1	172	59.9	
Normal	162	51.8	151	48.2	
Nuts (almonds, cashews, walnuts, etc.)					0.031
Less than normal	41	36.9	70	63.1	
Normal	236	48.3	253	51.7	
Beans (tofu, chickpeas, chili, etc.)					Constant
Less than normal	277	46.2	323	53.8	
Normal	0	0.0	0	0.0	
Dairy (cheese, milk, yogurt, etc.)					0.060
Less than normal	137	50.4	135	49.6	
Normal	140	42.7	188	57.3	
Eggs (omelet, in salad, etc.)					Constant
Less than normal	277	46.2	323	53.8	
Normal	0	0.0	0	0.0	
Grains (bread, pasta, rice, etc.)					Constant
Less than normal	277	46.2	323	53.8	
Normal	0	0.0	0	0.0	
Sweets (candy, cookies, pie, etc.)					0.425
Less than normal	243	45.6	290	54.4	
Normal	34	50.7	33	49.3	
Caffeinated soft drinks (cola, diet cola, energy drinks, etc.)					0.529
Less than normal	270	46.4	312	53.6	
Normal	7	38.9	11	61.1	
Coffee and tea (hot coffee, iced coffee, black tea, etc.)					0.536
Less than normal	42	43.3	55	56.7	
Normal	235	46.7	268	53.3	

sweets, and taking caffeinated soft drinks, drinking coffee, and tea.

Furthermore, the present study showed that there was no significant ($P > 0.05$) association of severity of anemia with eating beef, which was consistent to the results of Aikawa *et al.*,[11] who found that there was no significant association of Hb level with consumption of meat and meat products.

Results of the present study showed that eating beef less than normal had 0.688 times more likely to express risks of anemia than in nonanemic, which was consistent to the results

Table 7: Association of the severity of anemia with dietary daily amounts of the study sample (n=277)

Variables	Moderate		Mild		*P*-value Chi-square test
	F	%	F	%	
Fruit (apples, bananas, oranges, etc.)					0.090
Less than normal	10	50.0	10	50.0	
Normal	81	31.5	176	68.5	
Vegetables (carrots, mushrooms, potatoes, etc.)					0.027
Less than normal	79	36.1	140	63.9	
Normal	12	20.7	46	79.3	
Chicken (fried chicken, in soup, grilled chicken, etc.)					Constant
Less than normal	91	32.9	186	67.1	
Normal	0	0.0	0	0.0	
Fish and seafood (tuna, shrimp, crab, etc.)					Constant
Less than normal	91	32.9	186	67.1	
Normal	0	0.0	0	0.0	
Beef (steak, meatballs, etc.)					0.326
Less than normal	34	29.6	81	70.4	
Normal	57	35.2	105	64.8	
Nuts (almonds, cashews, walnuts, etc.)					0.007
Less than normal	6	14.6	35	85.4	
Normal	85	36.0	151	64.0	
Beans (tofu, chickpeas, chili, etc.)					Constant
Less than normal	91	32.9	186	67.1	
Normal	0	0.0	0	0.0	
Dairy (cheese, milk, yogurt, etc.)					0.797
Less than normal	44	32.1	93	67.9	
Normal	47	33.6	93	66.4	
Eggs (omelet, in salad, etc.)					Constant
Less than normal	91	32.9	186	67.1	
Normal	0	0.0	0	0.0	
Grains (bread, pasta, rice, etc.)					Constant
Less than normal	91	32.9	186	67.1	
Normal	0	0.0	0	0.0	
Sweets (candy, cookies, pie, etc.)					0.217
Less than normal	83	34.2	160	65.8	
Normal	8	23.5	26	76.5	
Caffeinated soft drinks (cola, diet cola, energy drinks, etc.)					0.807
Less than normal	89	33.0	181	67.0	
Normal	2	28.6	5	71.4	
Coffee and tea (hot coffee, iced coffee, black tea, etc.)					0.521
Less than normal	12	28.6	30	71.4	
Normal	79	33.6	156	66.4	

of Abriha *et al.*,[12] who mentioned that consumption of meat was another factor which showed significant association with anemia in pregnant women.

El-Hindi[13] mentioned on their results of the study that there was a statistically significant difference ($P < 0.05$) between study populations with respect to nutritional, dietary status. Kefiyalew *et al.*[9] mentioned on their results that there was no significant ($P > 0.05$) association of eating red meat, poultry, fish consumption, fruit and vegetable consumption with anemia, which was in contrast to the results of the present study regarding eating red meat, vegetables, and chicken,

Table 8: Logistic regression analysis of the factors associated with anemia

Variables	*P* value	Odd's ratio	95% CI of OR
Vegetables (carrots, mushrooms, potatoes etc.)			
Less than normal	0.003	0.493	0.308–0.788
Normal (reference category)			
Chicken (fried chicken, in soup, grilled chicken, etc.)			
Less than normal	0.999	1.039	1.017–1.061
Normal (reference category)			
Beef (steak, meatballs, etc.)			
Less than normal	0.035	0.689	0.488–0.973
Normal (reference category)			
Nuts (almonds, cashews, walnuts, etc.)			
Less than normal	0.053	0.641	0.409–1.005
Normal (reference category)			

OR: Odds ratio, CI: Confidence interval

while it was consistent regarding eating fruits. Aikawa *et al.*[11] found on their results that there was no significant ($P > 0.05$) association of anemia with eating fruit which was consistent with the results of present study, while it was inconsistent regarding eating chicken, vegetable, meat, and meat products. Jufar and Zewde[14] found on their results that there was no significant ($P > 0.05$) association of anemia with drinking tea and coffee and taking fruit, which was consistent with results of present study, while it was in contrast regarding eating animal foods and green leafy vegetables. The results of Aikawa *et al.*[11] indicated that there was no significant ($P > 0.05$) association of Hb level with consumption of fish, seafood, and eggs, while there was a significant ($P < 0.05$) relation of consumption of beans with Hb levels. Khapre *et al.*[15] mentioned that there was no significant ($P > 0.05$) difference of degree of anemia and was found between vegetarian and non-vegetarian type of diet. Viveki *et al.*[16] found on their results that there was no significant association of severity of anemia with dietary habits such as vegetarian and mixed diets. Clinical practice guideline nutrition for pregnancy[10] described the food pyramid guidelines as the following: Starchy carbohydrates, such as whole grains and fiber-rich foods including breads, cereals potatoes, pasta, and rice, six or more servings a day from this group; where one serving is 1 bowl of cereal, 1 slice of bread or 1 medium potato, fruit and vegetables, at least five or more servings a day; 1 serving is 1 medium-sized fruit, for example, 1 apple or 3 dessert spoons of vegetables, dairy Foods which includes milk, cheese, and yogurt, 3 servings a day from this group; 1 serving is 125 g yogurt, 25 g of cheese or 200 ml milk, and protein foods including meat, poultry, fish, eggs or legumes, at least 2 servings a day: Where one portion is 50–75 g (2–3 oz) cooked meat, 100 g (4 oz) fish, 2 eggs or 6 dessert spoons beans. Murray and Mckinney[17] described the food plan and amounts per serving with recommendations intake for pregnancy as the following: Food and amounts/serving of whole grains (1 oz = 1 slice bread and ½ cup rice or pasta) for vegetables, fruits, and dairy group (1 cup milk or yogurt, 1.5 ounce hard cheese, and 2 cup cottage cheese) for protein group (1 ounce meat/poultry/fish, 1 egg, ¼ cup cooked beans, ¼ cup tofu, and 1 tablespoon peanut butter), while the recommended intakes for them as following: For whole grains 7–9 oz, vegetables 3–3.5 cup, fruits 2 cup, dairy group 3 cup, and protein group 6–6.5 oz.

Karaoglu *et al.*[18] conducted a cross-sectional study about the prevalence of nutritional anemia in pregnancy in an East Anatolian province, Turkey, who found that 40.2% of study sample consumed egg, 8.1% consumed red meat, poultry or fish, and 42.0% consumed fruit and vegetables daily. El-Hindi (2011) concluded in a study done regarding nutrition that 55.4% of the study sample get regular nutrition, while 44.6% get not regular nutrition in anemic group, in non-anemic group and 78.3% of study sample get regular nutrition while 21.7% get irregular nutrition during pregnancy. Results of a study done by Karaoglu *et al.*[18] showed that 19.4% of study samples ate one portion of red meat, poultry, and fish consumption every day, while 27.8% of them ate less than frequent of red meat, poultry, and fish consumption. Regarding eating fruit and vegetable consumption, 24.3% of participants ate one portion, while 29.1% of participants ate less frequents. These results are consistent with the results of the present study regarding eating chicken and vegetables, while they are in contrast regarding eating fruits and beef. Plante *et al.*[19] conducted a survey on Nunavik women about iron deficiency and anemia among women, they found that less than half 37.1% of the sample ate normal meat, which was consistent to the results of the present study, while it was in contrast regarding eating sweets, which indicated that less than half 39.4% of the sample ate normal sweets, on the other hand, it is also in contrast regarding drinking coffee and tea, which found that less than half 37.1% of the study sample drinking less than normal coffee and tea.

CONCLUSION

Eating less than normal of vegetables, beef, and nuts were indicated risks of anemia during pregnancy.

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Cihan University-Erbil Scientific Journal (CUESJ)

Research Article

Prevalence and Characterization of Hepatitis B and Hepatitis C Infection among Blood Donors in Erbil

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ABSTRACT

Blood transmitting infectious disease still remains a considerable global health problem. Hepatitis B virus (HBV) and hepatitis C virus (HCV) are two of the most commonly transmitted infectious agents. This prospective cross-sectional study was conducted between December, 2017, and February, 2018, at the Directorate of Blood Bank in Erbil Province, Northern Iraq. During that period, a total of 6173 blood donors donated blood; all blood donors were asked a series of questions through a structured questionnaire designed for such purpose. These patients were serologically examined for HBV and HCV. Positive blood samples were further analyzed serologically and confirmed by real-time polymerase chain reaction (RT-PCR). Among 6173 blood donors who were investigated for HBV, 7 (0.11%) and 98 (1.6%) were positive for hepatitis B surface antigen (HBs-Ag) and hepatitis B core Antibody (HBc-Ab), respectively, whereas during screening for HCV, 4 (0.06%) were positive for HCV-Ab. Coinfection (dual infection (HBV and HCV) was positive in 1 patient (0.01%). Among 98 reactive samples, 75.5% were positive for HBs antibody (HBs-Ab), the remaining 24 samples (24.5%) were regarded as occult hepatitis B infection (OBI), since they were positive for HBc-Ab, whereas negative both for HBs-Ag and HBs-Ab. The diagnosis of OBI could be confirmed by RT-PCR in 8 samples, 33% of samples. The overall incidence of HBV and HCV among examined blood donors was 0.5 %, and 0.06%, respectively. Amidst that incidence, 0.39 % were diagnosed as OBI. To prevent viral transmission through blood transfusion is needed to combine a different and sensitive method for HBV detection as well as evolve tests that have high sensitivity and specificity for serological markers. Moreover, a molecular tool that is sensitive enough to detect very low copies of viral DNA must also be developed.

Keywords: *Blood donors, hepatitis B surface antigen, hepatitis B surface antibody, hepatitis B, hepatitis C*

INTRODUCTION

Infections with hepatitis B virus (HBV) and hepatitis C viruses (HCV) are major global health problems worldwide: It is predictable that about 350 million persons are chronically infected with HBV, and nearly 200 million people are infected with HCV worldwide. Both hepatitis B and hepatitis C are among common infectious liver diseases worldwide.[1] Viral hepatitis is a systemic disease primarily affecting the liver, which induces acute inflammation of the liver, resulting in a clinical illness characterized by fever, gastrointestinal symptoms such as nausea, vomiting, and jaundice. Regardless of the virus type, identical histopathologic lesions are observed in the liver during acute disease.[2] Both HBV and HCV infections are known for their parenteral transmission; therefore, blood transfusion is a potential route of transmission.[3,4] The risk of infection with transfusion-transmitted viruses has been reduced remarkably since the introduction of serological screening; on the other hand, a zero-risk blood supply remains the ideal aim.[5] The risk of transfusion-transmitted HBV infection has been reduced by screening all blood donations for hepatitis B surface antigen (HBs-Ag) since 1970, screening for HBs-Ag is still regarded as an important tool for blood screening by the World Health Organization (WHO).[6] It was accepted that the disappearance of HBs-Ag indicates the clearance of HBV. Meanwhile, many reports pointed to the occurrence of post-transfusion hepatitis B despite the screening for HBs-Ag, necessitating the use of other markers for viral screening. For this, screening for HBc-Ab was recommended by the German Advisory Committee Blood in March 2005, especially for areas with low HBV prevalence.[7,8] Recently in the major blood bank of Erbil, HBc-Ab screening has also been performed for all blood donors. The donated blood with negative HBs-Ag but positive HBc-Ab will be

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discarded, these groups of blood donors are either infected but have been cured of the diseases, or else the donor is still infected with HBV that can be further confirmed and need to be further analyzed both serologically and by molecular detection using polymerase chain reaction (PCR).[9] The blood donors with HBs-Ag negative serology, HBc-Abs positive or negative and the presence of viral DNA by PCR are known as occult HBV infected occult hepatitis B infection (OBI). OBI is a risky group playing an important role in hepatitis virus transmission by blood transfusion.[10]

Regarding the HBV prevalence in the world could be divided into three areas where the prevalence of chronic HBV infection is high (>8%), intermediate (between 2 and 8%), and low (<2%).[11] Infection with Hepatitis B and C is regarded as a major cause of liver cirrhosis and hepatocellular carcinoma. The WHO estimates that 57% of cases of liver cirrhosis and 78% of primary liver carcinomas result from a hepatitis B or C virus.[12] Approximately 70%–80% of HCV-infected patients fail to clear the virus and develop chronic HCV infection, which is a risk factor for liver diseases such as fibrosis, cirrhosis, and hepatocellular carcinoma.[13,14]

Several studies have recorded the prevalence of HBV and HCV infection in blood donors of different Iraqi cities.[15-17] According to the studies that have been conducted in the past years investigating the seroprevalence of hepatitis B and hepatitis C, the prevalence rate in our region is low which is <2%.[18,19]

Lack of documentary data in Erbil governments and immigration of a significant number of refugees from a neighboring country to Kurdistan region, as well as foreign tourists and the traveling of Kurdish peoples to other countries, necessitate updated research on seroprevalence of infectious agents (HBV and HCV) among blood donors. Nonetheless, the HBV vaccine was added to the expanded program of vaccination in early 2000.[20] There was an absence of data on the success of the vaccination program; therefore, we thought of evaluating the antibody for these viruses in the blood which offer an idea of the level of protection against these infectious agents.

This study was conducted to find out the incidence of HBV and HCV among blood donors in Erbil community. Besides this, we further characterized the HBV infected individuals along with the screening for HBs-Ab, evaluating the level of protection against HBV infection among our community.

MATERIALS AND METHODS

Study Population and Sampling

This is a prospective and cross-sectional study investigating HBV and HCV among blood donors who donated blood in the Directorate of Blood Bank-Erbil, Iraq. All volunteers who visited the blood bank to donate blood from December 2017 to February 2018 were included in the study: Blood donors were selected after an interview through a structured questionnaire designed for such purpose. Blood donors with low or high hemoglobin concentration, high body temperature, having any disease, weight <50 kg, cupping in Past 6 months, operation in the last year, drinking alcohol, uncontrolled blood pressure, history of taking any drugs, fever, and having tattoos on their body were excluded in our study.

In total, 6173 participants donated blood during the specific time and were screened for HBV and HCV. Among those blood donors, 109 were reactive for HBV or HCV. Fifty healthy individuals were also included in this study as a control group. Each blood sample was collected in a 5 ml of a sterile tube, and then the whole blood was centrifuged, plasma was taken and stored at minus 20 freezer. The study was approved by the Ethics Committee at the Hawler Medical University, for dealing with the samples and participant information and a written informed consent was obtained from each blood donor for their participation in the study.

Detection of HBV Serological Markers

ELISA screening test for HBs Ag and anti-HBc

Monolisa™ HBs Ag ULTRA (Bio-Rad, France) assay is a one-step enzyme immunoassay based on the principle of the "sandwich" type using monoclonal antibodies and polyclonal antibodies selected for their capability to bind themselves to the numerous subtypes of HBs Ag.[21] Serologically, the samples were rechecked by Elecsys HBs Ag II by device Cobas e411(Roche, Germany). The Elecsys HBsAg II assay uses monoclonal and polyclonal anti-HBs antibodies (mouse and sheep) for the HBsAg determination. Monolisa™ Anti-HBc PLUS (Bio-Rad, France) is an enzyme immunoassay (indirect ELISA type) for the simultaneous finding of total antibodies to HBV core.[22]

Chemiluminescent immunoassay (CLIA) screening test for anti-HBs

CLIA way was used for detecting antibody of HBs by the Liaison XL (DiaSorin, Italy). Amid numerous enzyme assays that give light-emitting reactions, one of the most successful tests is the improved CLIA regarding a horseradish peroxidase labeled antibody or antigen and a mixture of the chemiluminescent substrate, hydrogen peroxide, and enhancers.[23]

Detection of HCV serological markers

Monolisa™ Anti-HCV PLUS Version 2 or 3 (Bio-Rad, France) is an indirect qualitative enzyme immunoassay for the detection of infection by the HCV based on the detection of anti-HCV antibodies in serum or human plasma.[24]

Detection of HBV DNA (PCR)

The extraction of HBV DNA is done by a kit EZ1 Virus Mini kit (v2.0 Qiagen, Germany) using the EZ1 Advanced XL device (QIAGEN, Germany).[25] The *artus* HBV RG PCR Kit is used in the automated system for the detection of HBV DNA using the PCR on Rotor-Gene Q (QIAGEN, Germany).[26] This is conducted for 24 samples that are negatives for anti-HBs and positives for anti-HBc IgG, and six samples are used as a negative control.

Statistical Analysis

Microsoft Office Excel and Statistical Package for the Social Sciences (SPSS) are used for data entry and analysis.

RESULTS

Among 6173 participants, 109 were reactive for HBV and HCV. Fifty negative samples were also included in this study as a control group for further analysis. Among the 159 participants

who were included in this study as a test and control group, the mean and standard deviation in age for the control group was 35.5 ± 8.6 and that for the test group was 40.9 ± 12. The ages of blood donors both for test and control group were grouped into six groups [Tables 1 and 2]. The minimum age is 19 years, and the maximum age is 73 years for the test group and 22 and 57 years for the control group.

Prevalence of Hepatitis B and Hepatitis C

Serologically, based on detection of HBs-Ag, HBc-Ab, and HCV-Ab, we decided whether the patient has HBV or HCV infection. These tests were carried out for all the participants (6173). Among all examined participants, only 7 patients were reactive for HBs-Ag (0.11%), whereas 98 donors were reactive for HBc-Ab (1.58%). Regarding HCV infection only 4 patients were positive for anti-HCV Ab (0.065%), as described in Figures 1 and 2.

Immune Blood Donors for HBV Infection

Among the sero-reactive blood donors for HBV, we further characterize them through detection of antibody against HBs-Ag (HBs-Ab). Should individuals of HBc-Ab positive blood have HBs-Ab in their blood, this indicates that these participants are cured of HBV infection and means that they have immunity to HBV infection. Among the 98 sero-reactive blood donors, 74 blood donors were positive for HBs-Ab (75.5 %) indicating that they are cured of natural infection.

We also examined 50 healthy volunteers that are free from HBV and HCV infection, among them only 4 blood donors were positive for HBs-Ab, reflecting the successful vaccination and protection against HBV infection [Table 3].

Diagnosing Occult HBV Infection

As we defined OBI before, blood donors with HBs-Ag negative serology, HBc-Abs positive or negative, and the presence of viral DNA by PCR are known as occult HBV infection (OBI). Blood donors with only positive HBc-Ab in the absence of HBs-Ag and HBs-Ab are regarded as OBI.[10] In our study, we further characterize the positive blood donors. Among the samples that are included in our study, 98 samples were positive for HBc-Ab, from which 24 blood donors (24.5%) were negative for HBs-Ag and HBs-Ab (HBc-Ab +ve, HBs-Ag –ve, and HBs-Ab –ve), indicating that these blood donors were defined as having occult hepatitis virus infection [Table 3]. These 24 samples were further investigated for the detection of HBV DNA [Figure 3].

HCV Incidence and Dual Infection

In this study, as we mentioned above, besides screening for HBV infection, screening for HCV was also done for these participants. Among 6173 participants, only four blood donors were positive for HCV-Ab 0.06%. Among HCV reactive donors, one individual was positive both for HBV infection and HCV infection, which means that he has a dual infection [Table 4].

Sero-status in Relation to Socio-demographic Characteristics

Among the HBV and HCV positive blood donors, the highest frequency of 38 (35%) was found among those aged between 26 and 35 years (young age groups). Blood donors with blood Group O were found to have the highest frequency for

Table 1: Demographic distribution of the HBV and/or BCV positive samples (positive blood donors)

Demographic distribution	Frequency (*n*=%)
	Test group
Age groups (year)	
Under 25	4 (3.7)
2–35	38 (35)
36–45	33 (30)
46–55	22 (20)
56–65	7 (6.4)
Above 66	5 (4.6)
Blood groups	
O	39 (36)
A	36 (33)
B	25 (23)
AB	9 (8)
Occupations	
Private sector employee	73 (67)
Public sector employee	30 (27.5)
Unemployed	6 (5.5)
Student	-
Total	109 (100)

Table 2: Demographic distribution of the samples control group (negative blood donors)

Demographic distribution	Frequency (*n*=%)
	Control group
Age groups (year)	
Under 25	6 (12)
26–35	25 (50)
36–45	10 (20)
46–55	8 (16)
56–65	1 (2)
Above 66	-
Blood groups	
O	17 (34)
A	18 (36)
B	10 (20)
AB	5 (10)
Occupations	
Private sector employee	34 (68)
Public sector employee	13 (26)
Unemployed	1 (2)
Student	2 (4)
Total	50 (100)

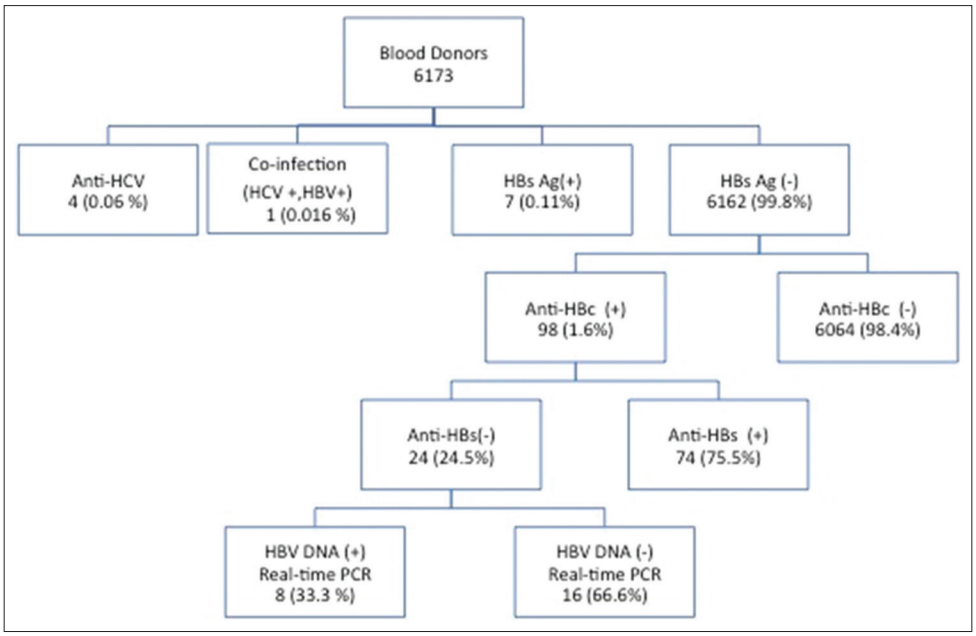

Figure 1: Serological and molecular characterization of hepatitis B virus and hepatitis C virus infection among blood donors in the major blood bank of Erbil city. HBs Ab – hepatitis B surface antibodies; HBc Ab – hepatitis B core antibodies: PCR – a polymerase chain reaction

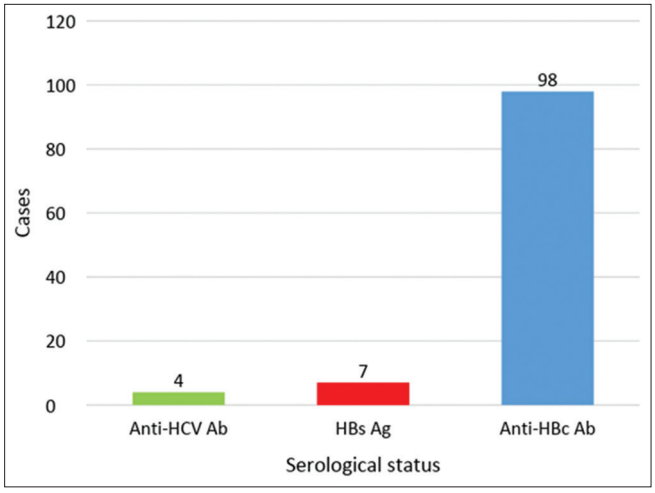

Figure 2: Incidence of both hepatitis B virus and hepatitis C virus among blood donors

its immunoreactivity among test group 39 (36%) [Table 1], whereas those with blood Group A are among those with the highest frequency among control group 18 (36%) [Table 2]. Most of the reactive individuals for HBV positivity were among the private sector employees [Table 1]. None of these parameters were statistically significant.

Molecular Detection of the HBV by Real-time Quantitative PCR (RT qPCR)

For further characterization of serum samples from HBs-Ag negative, HBs-Ab negative, and HBc-Ab positive blood donors were processed for HBV-DNA detection using molecular amplification using the RT PCR. This test was done using artus® HBV RG* PCR Kit 24, V1 (Qiagen, Germany). After extracting the nucleic acid of the HBV by EZ1®Virus Mini Kit v2.0 (Qiagen, Germany), we ran the purified DNA of HBV by Rotor-Gene Q (Qiagen, Germany). Twenty-four samples were selected (Anti-HBc IgG positive and negative for anti-HBs), and six control samples (negative control) were also included in the study. Out of these samples, 8 of the samples (33%) were positive, showing the DNA in their blood, confirming the diagnosis of occult hepatitis B [Figure 3].

DISCUSSION

Hepatitis B and C viruses are responsible for causing about 80% of liver cancer cases worldwide, and it could be the major cause of morbidity and mortality.[27] Regarding hepatitis prevalence, the world is divided into three areas; low prevalence area, which means <2%, an intermediate area 2–8% and high prevalence area more than 8%.[28,29] Most countries on the earth are still viewed as intermediate to high endemicity for HBV infection.[28,29] HCV follows the same division regarding prevalence such as low prevalence (<1.5%), moderate prevalence (1.5%–3.5%), and high prevalence area, which is more than 3.5%.[30]

The current study characterizes and defines the frequency of HBV and HCV (6173) blood donors. For HBV detection, two serological markers were used (HBs-Ag and Hbc-Ab), among those, 98 donors were reactive for HBc-Ab (1.58%), whereas only 7 people were positive for HBs-Ag (0.11%). For screening of HCV, HCV-Ab was used and only 4 blood donors proved positive for anti-HCV-Ab (0.065%), as described in Figures 1 and 2.

The prevalence of reactive blood donors in the current study for HBV is 1.7% (105 out of 6173) from which 1.5% is reactive against HBc-Ab and 0.11% is reactive against HBs-Ag, whereas for HCV is 0.06%. They are in agreement on a national level with these results by Alhilfi *et al.,* who screened 36620 blood donors in Missan Governorate in Iraq,

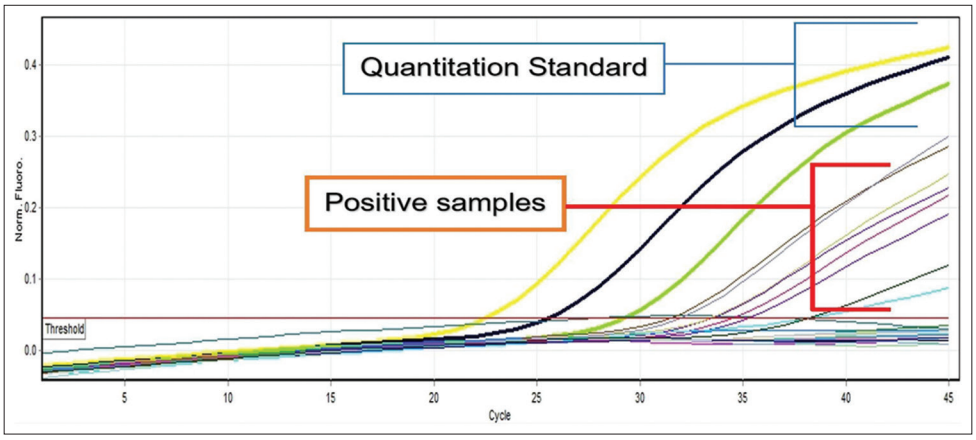

Figure 3: Quantitation data for cycling A. Green for hepatitis B virus by real-time quantitative polymerase chain reaction

Table 3: Distribution of HBs-Ab among HBc-Ab positive individuals and control group

Serology (HBc IgG)	Serology (HBs antibody)			*P*-value
	Positive (*n*=)	Negative (*n*=)	Total (*n*=)	
Positive	74	24	98	<0.05
Negative	6	55	61	
Total	80	79	159	

Hbs Ab: Hepatitis B surface antibodies

Table 4: The incidence and percentage of HCV reactive donors and dual infection

Serology (HBs antigen)	Serology (HCV-Ab)		
	Positive	Negative	Total
	n= %	*n*=%	*n*=%
Positive	1 (0.6)	7 (4.4)	8 (5.0)
Negative	3 (1.9)	148 (93.1)	151 (95.0)
Total	4 (2.5)	155 (97.5)	159 (100.0)

HCV-Ab: Hepatitis B surface antibodies

they found 0.12% positivity for HBs-Ag and 0.09% positivity for HCV-Ab.[31] Similar results were also found by Al-Rubaye *et al.,* who screened 69915 blood donors in Basra in 2013, from which they found 125 reactive individuals for HBs-Ag (0.2%), and 1475 reactive individuals for HBc-Ab (2.1%) and HCV-Ab positivity in 0.1%.[17]

On an international level, similar results to ours were also found in a neighboring country by Boustani *et al.,* which was done in Ilam city in Iran between 2009 and 2013: They screened 72,527 blood donors, they found HBs-Ag in 102 (0.14%), and HCV-Ab in 27 (0.037%).[32] Moreover, another study conducted in Southwestern Iran, by Sajjadi *et al.,* they Screened 180,304 blood donors and found HBV in 0.13% and HCV in 0.06%.[33]

On the other hand, our data were much lower than that of other related studies performed in other centers. Nationally, Hussein *et al.* performed a study in Duhok at 2004 found HBs-Ag in 62/7900 (0.78%), and HCV-Ab in 16/7900 (0.2%).[15]

In addition, internationally, our result was lower than those found in India in a study conducted by Gulia, they found HBV in 2.54%.[34] In Libya, a study carried out by Qowaider *et al.,* they examined 78,987 blood donors in different regions in the Northeast in Libya, and they found HBV in 172 blood donors (0.21%), and HCV in 197 blood donors (0.24%).[35] Furthermore, in Turkey, Hope *et al.,* examined 53,985 blood donors, among them 2.1% were positive for HBV and 0.3% for HCV.[36]

Besides the blood screening, we further characterized the reactive blood donors (positive samples) among 105 positive samples for HBc Ab (1.7%), 98 samples were HBc-Ab positive but negative for HBs-Ag (HBs-Ag −ve/HBc-Ab +ve), further characterization was done for these group by investigating for HBs-Ab, among these 74 (75.5%) blood donors were HBs-Ab positive (HBc-Ab +ve, HBs-Ag –ve, and HBs Ab +ve) which indicated that they are cured of natural infection [Figure 1 and Table 3] whereas the remaining 24 blood donors (24.5 %) were HBc-Ab positive but negative both for HBs-Ag and HBs-Ab. Individuals with negative HBs-Ag and HBs-Ab but positive HBc-Ab are defined as occult HBV infection (OBI), this is because HBc-Ab is regarded as a pathognomonic marker of HBV infection, particularly when HBs-Ag and HBs-Ab are negative.

We performed HBV-DNA detection assay solely on samples of blood donors who tested positive for HBc-Ab but negative for both HBs-Ag and HBs-Ab (24 samples), including six control samples as well. Among 24 samples we could confirm the presence of HCV DNA only in 8 (33%) samples, using RT PCR, indicating the overall incidence of OBI by DNA detection to be about (0.13 %; 8 out of 6173) [Figures 1 and 3]. Based on the reaction graph, most of the positive sample has very low copy numbers since the reaction was arrived at between the 35 and 40 cycles [Figure 3].

Meanwhile, the remaining 16 (66 %) samples were HBc-Ab positive but failed to be confirmed by HBV-DNA

detection. In most of the areas, these groups of blood donors are still regarded as OBI, and their blood will be discarded to prevent virus transmission.[37]

Internationally, many studies have been published throughout the world showing the frequency of OBI within HBs-Ag-negative donors, the results are varied: For example in the United Kingdom it was 0.56%;[38] in the United State of America it was 0.8%;[39] in Iran it was 2.1%;[40] and in Brazil it was 3.1%.[41]

On a national level, a similar study performed in Erbil City of Iraq shows OBI in 0.09% which is close to our finding (0.13%).[42] Discarding the infected blood on the basis of HBc-Ab detection, especially in areas where the prevalence of HBc-Ab is high (more than 10%), will negatively affect the blood supply. In the present study, we found that the incidence of HBc-Ab in the Erbil city was low (1.56%, 98 out of 6173) [Figure 1]. Further detection of HBs-Ab saved 78 of our samples (75.5%) since they were positive for HBs-Ab. On the other hand, DNA confirms the OBI only in 8 samples (33%). In high prevalence area based on HBc-Ab detection such as Korea, Greece, and Ghana, they discarded only blood that is positive for HBV-DNA, consequently saving more blood units.[43,44] Meanwhile, in many places, especially in low prevalence countries, blood donor samples were still considered as OBI based on HBC-Ab positivity, and all such donated blood was discarded to prevent transfusion-transmitted viral infection.

Despite the above, the failure of detecting HBV-DNA might be due to a mutation in viral structure, and the false serological result might be due to the imperfection of the assays used for such purpose.[37]

Many researchers argued in favor of the implementation of HBc-Ab or HBV DNA alone or combination for the screening of donated blood.[45,46] In a large study screening, 6.5 million participants using three markers (HBs-Ag, HBc-Ab, and PCR for HBV DNA), prioritizing the use of HBc-Ab along with the DNA nucleic acid testing was confirmed for the detection of a low level of HBV DNA positive donors.[46]

CONCLUSION

The overall incidences of HBV and HCV among examined blood donors were 0.5% and 0.06%, respectively. Among that, 0.39% were diagnosed as OBI. The highest avoidance of HBV blood transmission can be attained by combining a different, more sensitive, method for HBV detection with the evolving of tests that have high sensitivity and specificity for HBs Ag and HBc Ab detection. Further, developing a molecular tool that is sensitive enough to detect a very low copy of viral DNA would conserve many pints of blood.

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CONFLICTS OF INTEREST

There are no conflicts of interests to be declared.

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Research Article

Significance of Hepatitis B Virus Diagnosis by Real-time Polymerase Chain Reaction over Serological Markers in Hepatitis B Virus Patients

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ABSTRACT

Hepatitis B virus (HBV) is the leading cause of viral hepatitis, as currently over 2 billion people have HBV infection worldwide. Nucleic acid assay and quantitative hepatitis B surface antigen (HBsAg) have been developed for diagnostic and therapeutic monitoring of patients with HBV infection. These tests might also show correlation between HBV DNA and HBs serostatus. The study aimed to find and analyze the frequency and impact of HBsAg seropositivity among patients revealed HBV DNA negative level through quantitative estimation of both seromarkers. Real-time polymerase chain reaction (RT-PCR) and Elecsys assays were used for quantitative estimation of HBV DNA and HBs antigen, respectively. A total of 256 blood samples were used from patients referred for either diagnostic purpose and/or HBV viral load monitoring after antiviral therapy. Blood profile analysis showed 12.26% HBs antigen seropositivity among patients revealed negative for nucleic acid assay for HBV DNA. Positive HBs antigen titers ranged from 1000–50,000 COI, with seronegative anti-HBs antibody test for all samples tested positive for HBs antigen. This study delineated that negative or undetectable quantitation of HBV DNA level does not exclude HBV infection; as the level might fluctuate in different phases of HBV replication. This gives an impression and raising a question about significance of replacing test for HBsAg with quantitation of HBV DNA PCR assay. Thus, the study refers to a special HBV profile outside the classical pattern.

Keywords: Component, hepatitis B surface antigen, hepatitis B virus, hepatitis, quantitative hepatitis B virus DNA

INTRODUCTION

Hepatitis B virus (HBV) is the leading cause of viral hepatitis, as currently over 2 billion people have HBV infection worldwide. Approximately 600,000 deaths occur every year as a result of the acute and chronic consequences of HBV infection.[1] Chronic HBV affects nearly 350 million patients worldwide and may further progress to cirrhosis and/or hepatocellular carcinoma (HCC) in 15–40% of cases.[2] HBV infection is a dynamic process characterized by replicative and non-replicative phases based on virus-host interaction, which are present in some form of all infected patients.[3] To measure HBV DNA (viral load), a laboratory measures how many HBV DNA units are found in 1 ml of blood. The result is written in international units per ml (IU/ml). High levels of HBV DNA indicate a high rate of HBV replication; however, low or undetectable levels–<2000 IU/ml indicate an "inactive" infection.[4] During HBV infection, markers can be detected in sera samples using immunoassays. For example, patient sera that contain anti-HBs indicates immunity from past infections or vaccinations, and a patient with anti-HBe antibodies in their sera is considered as spontaneous resolution of infection or therapy-induced improvement.[5,6] One of the serological methods using for the diagnosis of HBV is enzyme immune assays. The hepatitis B surface antigen (HBsAg) and HBV DNA levels were considered to be a risk factor of hepatocarcinogenesis in untreated HB patients.[7] The levels of quantified HBsAg are considered as a marker with which to evaluate the host immunological control of infection and HBV replication.[8] Low HBsAg levels in some HBV patients are considered to indicate a high likelihood of HBV clearance and lower hepatitis activity.[7,9] The aim of this study was to estimate HBV-DNA positivity states among serologically screened patients and to compare the sensitivity of molecular and serological methods for the diagnosis of HBV infections.

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MATERIALS AND METHODS

For this cross-sectional study, a total of 256 blood samples were collected from HBV patients referred to Erbil Central Laboratory, from June 2017 to September 2017. Both sexes were included (154 males and 102 females), with age range between 20 and 60 years. Real-time polymerase chain reaction (RT-PCR) as a molecular and Elecsys assay as a serological method were used for the diagnosis and comparing their sensitivity purposes. For RT-PCR technique, 5 ml of venous blood were collected using EDTA tubes and centrifuged at 1000 rpm for 5 min and patients' plasma was separated. Viral DNA was extracted from a 200 μl aliquot of serum using a Qiagen mini blood kit (Qiagen, Hilden, Germany) according to the manufacture instructions. Viral loads of HBV DNA were estimated by RT-PCR (HBV RG PCR artus, Germany). The amplification was performed in a 50 μl reaction mixture containing 30 μl of mixture of HBV RG Master mix (buffer, dNTP, primer, probe, and enzymes) and HBV RG inhibition control mixed with 20 μl of DNA template to each reaction. The primers HBV-Taq I (forward primer): CAA CCT CCA ATC ACT CAC CAAC and HBV-Taq 2 (reverse primer): ATA TGA TAA AAC GCC GCA GAC AC were used from the sequences of the conserved regions of the HBV surface gene used previously by Weinberger *et al*.[10] For performing RT-PCR, Rotorgene 3000 (a machine used routinely in Erbil Central Laboratory for detection of variety of DNAs and RNAs) was used following manufacture's instructions. The RT-PCR cycling parameters consisted of denaturation at 95°C for 15 s, 55°C for 30 s, and 72°C for 15 s. The results were considered positive if a signal was detected in Cycling A FAM, whereas no signal detection indicated as negative results. The quantitation of HBV DNA was performed for all blood samples and the results were considered positive and significant if viral load was more than 1×10^5 viral copies/ml. Titers <50 IU were considered as negative for HBV DNA. The screened results were categorized according to positive HBV DNA titers into inactive, gray zone, and active carriers.

Quantitation of HBsAg was measured using the Elecsys HBsAg quantitative assay. Detection of HBsAg by Elecsys utilizes a sandwich principle and this assay has two stages: First, a complex is formed with two monoclonal HBsAg-specific antibodies, one of which is biotinylated and the other labeled with a ruthenium complex. This complex joins to the solid phase through the interaction of biotin and streptavidin after the attachment of streptavidin-coated microparticles.[11] The mixture is subsequently aspirated into a measuring cell, where application of a voltage induces chemiluminescent emission, which is measured by a photomultiplier. All serum samples were tested at a dilution of 1:400. The Elecsys HBsAg quantitative assay is calibrated to give results in IU/ml.[11]

Statistical Analysis

Statistical analysis was done using SPSS 23.0. HBV titers expressed as means ± standard error. Comparisons between active and inactive carriers use *t*-tests. Comparisons between multiple groups were performed by ANOVA. $P \leq 0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

Of the 256 cases, 154 (60%) were male and 102 (40%) were female, with age range between 20 and 60 years. RT-PCR results showed that 163 of 256 samples were HBV DNA negative, from them, 20 samples showed positive results for HBsAg quantitation [Table 1].

The Asian Pacific Association for the Study of the Liver, European Association for the Study of the Liver, and the guidelines of the American Association for the Study of Liver Diseases refer that the disappearance of HBsAg is the ideal and cornerstone during natural course of HBV infection.[12-14] From the clinical standpoint of view, the progression from chronic hepatitis B to cirrhosis is increasingly signified through the HBV viral load.[8] Meanwhile, quantitative HBsAg level was regarded as marker for clearance, hepatitis activity, and immunological host response to control HBV replication and the likelihood of inactive virus carrier.[9,7,15] However, little is known about correlation between HBV replication and low levels of HBsAg, as reported in some patients with chronic HBV infection.[16-18]

The results of the present study showed 12.26% HBs seropositivity (range 1000–5000 COI) among patients seronegative for HBV DNA [Table 2], with highly significant difference ($P \leq 0.001$) between mean of HBV DNA seropositive

Table 1: Baseline patient's characteristics

Variables	All patients (*n*=256)
Gender	
Male	154 (60%)
Female	102 (40%)
HBV DNA IU/ml	256
<50 (negative)	163 (63.67%)
101–500	22 (24.44%)
501–1000	10 (11.11%)
1001–2000	10 (11.11%)
2001–20,000	20 (22.22%)
>20,000	31 (34.44%)
qHBsAg tested	163/256 (63.67%)
<10 COI	143 (55.85%)
1000–2000 COI	3 (15%)
2001–3000 COI	4 (20%)
3001–4000 COI	8 (40%)
4001–5000 COI	5 (25%)
Anti-HBsAb tested	20
Positive	0.00
Negative	20 (100%)
HBs positive, HBV DNA negative	20/163

qHBsAg: Quantitative hepatitis B surface antigen, HBV: Hepatitis B virus

Table 2: Frequencies of HBV DNA seropositivity among patients negative for HBV DNA assay

Number screened	HBV DNA PCR +ve	HBV DNA PCR−ve (*n*=163)	
		HBs +ve	HBs −ve
n=256	93 (36.32%)	20 (12.26%)	143 (87.73%)

HBV: Hepatitis B virus, PCR: Polymerase chain reaction

and negative titers [Table 3]. Regarding to the gender positivity for PCR test, in total of 154 male patients 65 (42%) were positive and of 102 Female patients only 28 (27%) were positive [Table 4]. The seromarkers profile among patients negative for HBV DNA revealed anti-HBs antibody seronegative among the total 20 patients positive for HBsAg [Table 5].

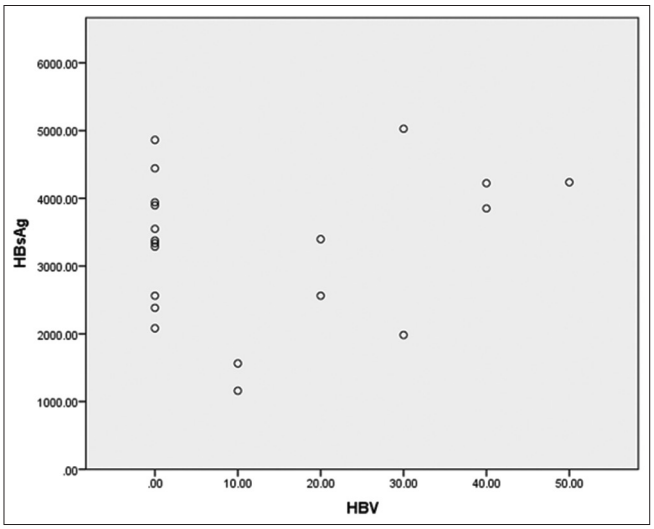

Figure 1: Pearson correlation between quantitative hepatitis B virus DNA level and hepatitis B surface antigen. $R = 0.229$, $P = 0.331$

Table 3: Mean titers of HBV DNA among patients screened

Number screened	HBV DNA PCR +ve (*n*=93)	HBV DNA PCR −ve (*n*=163)	*P*-value
n=256	18,582,661.24±5,934,488.97	31.12±4.45	0.000

HBV: Hepatitis B virus, PCR: Polymerase chain reaction

Table 4: Frequencies of HBV DNA positivity according to the gender

Gender	Positive PCR	Negative PCR	Total	*P*-value
Male	65 (42.20%)	89 (57.79%)	154	≤0.001
Female	28 (27.45%)	74 (72.54%)	102	
Total	93 (36.3232%)	163 (63.67%)	256	

HBV: Hepatitis B virus, PCR: Polymerase chain reaction

However, results of anti-HBs titers among patients negative for HBV DNA showed 20 samples were positive showing different titers [Table 6]. During the immune-tolerant phase, HBsAg level is the highest one, and the level decreases progressively from immune tolerance to inactive phase that reflects a low replicative phase of immune control.[19] The detection of HBV infection is vital for diagnosis our group published a paper that delineated the impact of PCR DNA assay for the diagnosis of HBV infection.[20]

The data of this study are agree with other studies[21] for the serologic profile of HBsAg seropositivity among patients screened negative for HBV DNA. They reported 6% of their samples gave HBsAg positive among donations negative for HBV DNA level, others[8] referred that in nine patients with HCC; HBV DNA was undetectable but the level of HBsAg was ≥2000 COI. Others reported 31.4% HBsAg positive among patients negative for HBV DNA.[22] However, data from another study showed that 100 of 140 patients were seronegative for HBV DNA.[23] Another study[24] reported that 69% of confirmed HBsAg-positive donations had undetectable HBV DNA levels. However, others[25,26] found that none of the HBsAg-positive donors with very low or negative for HBV DNA were anti-HBs antibody positive.[24,27]

Among explanation for such unusual HBV serologic profile, the patients may be chronically infected with HBV particularly; they are in the non-replicative phase as part of the natural course of infection.[22] The question might be raised here is about such serologic profile of HBsAg positive among patients negative for HBV DNA, are these patients infectious and transmissible for community,[21] referred for the need of larger sample volume (5–10 ml) necessary for extraction and concentration to determine the real HBV DNA status among samples negative for DNA.

In this study, Pearson's correlation analysis revealed no significant correlation between serum HBV DNA and HBsAg levels ($r = 0.331$; $P > 0.3$) [Figure 1]. Different studies reported discrepant results regarding correlation between HBV DNA and HBsAg levels, our data were disagree with other studies who reported positive correlation.[28-34] Meanwhile, our results agree with others for no correlation between HBV DNA and HBsAg levels.[35,36] Such correlation is only weak in patients negative for HBsAg.[19,30,37,38] This is because the level of HBV DNA fluctuates from undetectable to >2,000,000 IU/ml.[12] This is why the level of HBsAg does not fall in the same proportion or level when correlated with HBV DNA level as a marker for

Table 5: HBsAg and anti-HBs seromarkers profile among patients negative for HBV DNA

Serological profile	HBs		Anti-HBs (*n*=20)	
	>10 COI +ve	<0.9 COI −ve	<10 IU/ml −ve	>10 IU/ml +ve
HBV DNA PCR negative (*n*=163)	20 (12.27%)	143 (87.73%)	20 (100%)	0.00

HBV: Hepatitis B virus, PCR: Polymerase chain reaction, HBsAg: Hepatitis B surface antigen

Table 6: Distribution of HBsAg and anti-HBs titers among patients negative for HBV DNA

HBs titers COI					Anti-HBsAb IU/ml	
Non-reactive <0.9 COI	1000–2000	2001–3000	3001–4000	4001–>5000	Non-reactive <10	Reactive >10
0.00	3 (15%)	4 (20%)	8 (40%)	5 (25%)	20 (100%)	0.00

HBV: Hepatitis B virus, PCR: Polymerase chain reaction, HBsAg: Hepatitis B surface antigen

disease progression.[4] Martinot-Peignoux *et al*.[39] referred that HBV DNA diminished with time from seroclearance; however, covalently closed circular DNA as integrated HBV DNA remained detectable in liver biopsies. It is thus critical for clinicians to be aware of the significance of HBV DNA serial measurement and lifelong follow-up to establish and confirm that inactive carrier condition is maintained.[4] Among major guidelines, if HBV DNA is undetectable, either prophylaxis with lamivudine or strict follow-up every 1–3 months for ALT and HBV DNA level is indicated.[13]

CONCLUSION

Serology will undoubtedly continue to be widely used in the detection of HBV infection; however, HBV DNA assays can serve as an important tool, especially in detecting low levels of HBV DNA and in patients with the past HBV infection. Data from the present study also concluded that negative or undetectable quantitation of HBV DNA level does not exclude HBV infection; as the level might fluctuate in different phases of HBV replication. This gives an impression and raising a question about significance of replacing test for HBsAg with quantitation of HBV DNA PCR assay. Thus, the study refers to a special HBV profile outside the classical pattern.

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Cihan University-Erbil Scientific Journal (CUESJ)

Research Article

Radish Juice Promote Kidney Stone Deposition in Ethylene Glycol-induced Urolithiasis in Rats

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ABSTRACT

Urolithiasis is a well-known problem that stones could form in various parts of the urinary system and it is the most common disease of the urinary tract. The current study was planned to investigate the effect of radish juice on ethylene glycol (EG)-induced urolithiasis. Twenty-one rats randomly divided into three groups. The first group was the control group was received normal standard diet and drinking water and the second group represented the model group received 0.75% EG in drinking water *ad libitum*. The third group received radish juice (2 ml/kg of body weight) by gavage plus EG (0.75%) in drinking water *ad libitum*. The experiment was conducted for 28 days. The light microscope examination revealed a disturbed histological architecture of the kidney tissues, including dilated renal tubules, aggregation of infiltrated leukocytes inflammatory cells, and crystal deposition in the model group. The EG plus radish juice treated rats showed higher crystal density with improved renal tubule structure and alleviated inflammation. Both treated groups showed various biochemical alterations compared to control group, but the most interest biochemical result was the significant decrease of malondialdehyde, a lipid peroxidation marker, and in the radish plus EG group compared to the EG group. Scanning electron microscopy showed clear structural detail about calcium oxalate crystals in which radish-treated group showed higher crystal deposition and calcified tissue compared to EG group. The present study concluded that radish juice promotes stone deposition but exerted an antioxidant effect.

Keywords: Ethylene glycol, kidneystone, radis, urolithiasis

INTRODUCTION

Urolithiasis is a well-known problem that stones could form in various parts of the urinary system.[1] Mankind has been afflicted by urinary stones for centuries dating back to 4000 B.C. and it is the most common disease of the urinary tract.[2] Urolithiasis affects about 12% of the world population at some stage in their lifetime.[3] Urolithiasis is a complex process that occurs due to imbalance between promoters and inhibitors.[4] The chemicals that can promote stone formation are calcium, sodium, oxalate, urate, and cystine, while magnesium, citrate, and pyrophosphate can inhibit the urolithiasis process.[5] Calcium containing stones are the most common renal stones with a percentage of 80% of cases.[6] The process of stone formation begins with supersaturation that is the driving force for crystallization in solutions like urine.[7] As a result of supersaturation, solutes precipitate in urine leads to nucleation and then crystal growth. Crystallization happens when chemical concentrations exceed their point of saturation.[8] Then after, small hard mass of crystals sticks to each other to form a larger stone that is called crystal aggregation.[7] Plants such as *Oxalis corniculata,*[9] *Phyllanthus niruri,*[10] *Bergenia ligulate,*[11] and *Nigella sativa*[12] have been used as a remedy for renal calculi. Radish (Raphanus sativus) is a member of the family Cruciferae. This family consists of annual, biennial, or perennial herbs with pungent oils in the sap. There are about 380C Genera and about 3000 species.[13] Varieties of radish are now broadly distributed around the world, but there are almost no archeological records available to determine its early history and domestication.[14] Radish has been shown medicinal benefits such as gastroprotective effects[15] as a remedy for diabetes treatment[16] and anti-microbial activity.[17] The present study has been designed to evaluation of protective effect of radish juice on renal stone.

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MATERIALS AND METHODS

Experimental Animals and Radish Juice Preparation

For conducting this experiment, 21 mature male rats were bred under standard animal house conditions. For radish juice preparation, radish obtained from local market in Erbil city, systematic identity confirmed in College of Science/ Salahaddin University, washed, cut to small pieces, and homogenized then filtered by gauze. The rats were divided randomly into three groups; each group consisted of seven rats per cage Group 1, control, the rats of this group were given standard rat chow and tap water for 28 *days*. Group 2, ethylene glycol (EG)-induced urolithiasis model. The rats of this group were given standard rat chow and EG (0.75%) in drinking water *ad libitum* for 28 days. Group 3, radish, the rats received standard rat chaw, radish juice (2 ml/kg of body weight) by gavage, and 0.75% EG in drinking water *ad libitum* for 28 *days*.

The animals were anesthetized by intraperitoneal injection of combination ketamine hydrochloride 80 mg/kg (Trittau, Germany) and xylazine 12 mg/kg (Interchem, Holland). After sacrification, kidneys removed then fixed in desired fixative according to the type of microscopical preparation.

Kidney Weight Recording

Both kidneys have removed, weighted, and expressed as gram per 100 *g* of body weight.

Urine Flow

Animals remained in urine collector and urine was collected for 24 *h* then urine flow measured as ml/h/kg.

Fluid Intake

Fluid intake recorded every 4 *day*s during the experiment.

Blood Collection

At the end of the treatment period, blood samples were collected from all anesthetized rats of four groups through cardiac puncture in which the collected blood samples were immediately placed into gel tube for serum collection, later were centrifuged (Hettich D-78532/Germany) at 3000 *rpm* for 15 *min*. The sera were stored at −80°C (Sanyo – Ultra-low Temperature, Japan) until assayed.

Serum Urea Determination

Urine and serum urea was determined by urea kit (BIOLABO, France). Enzymatic and colorimetric method of urea determination is based on hydrolysis of urea to ammonium and carbon dioxide by urease. Ammonium reacts with chloride and salicylate and makes a blue-green complex that is proportional to urea concentration and measured at 600 *nm*.

Estimation of Creatinine and Glomerular Filtration Rate

Serum-urine creatinine was determined spectrophotometrically using BIOLABO kit (France).

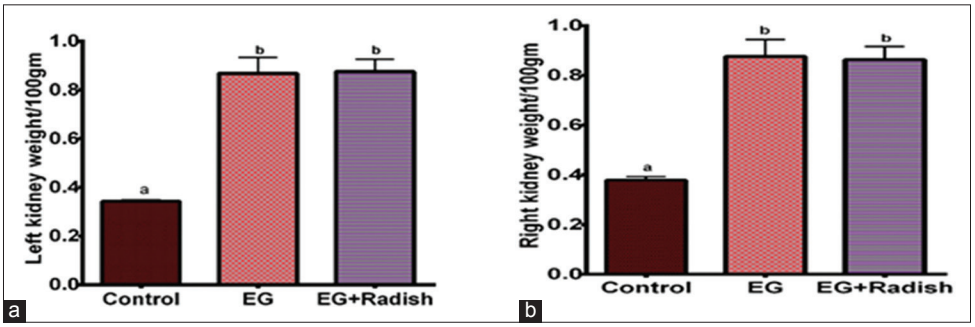

Figure 1: Left (a) and right (b) kidney weight per 100 g of body weight. Different letters on bars mean significant change and the same letters means no significant change

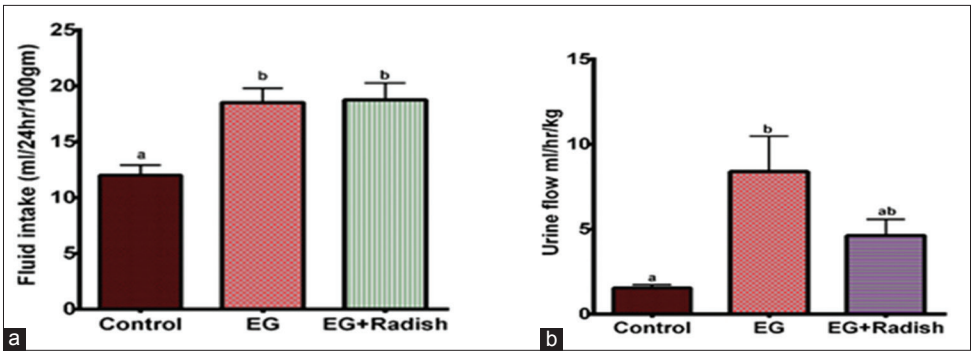

Figure 2: Fluid intake (a) and urine flow (b) of control and ethylene glycol treated groups. Different letters on bars mean significant change and the same letters means no significant change

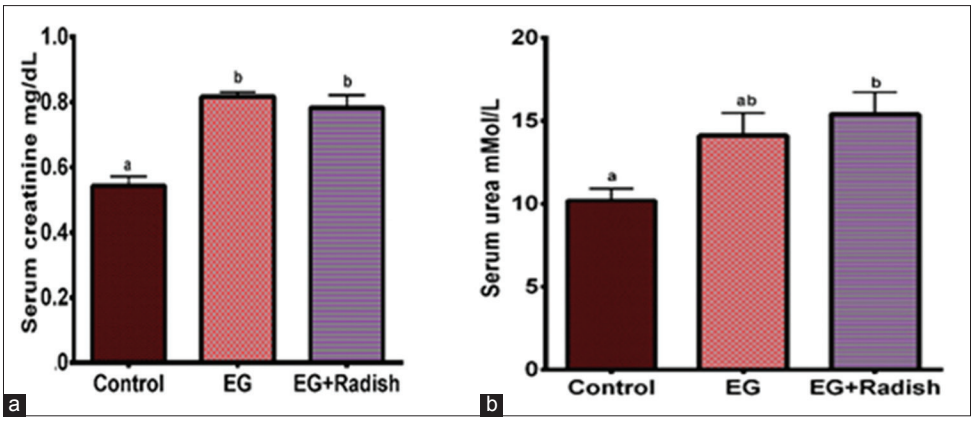

Figure 3: Serum creatinine (a) and serum urea (b) of control and ethylene glycol treated groups. Different letters on bars mean significant change and the same letters means no significant change

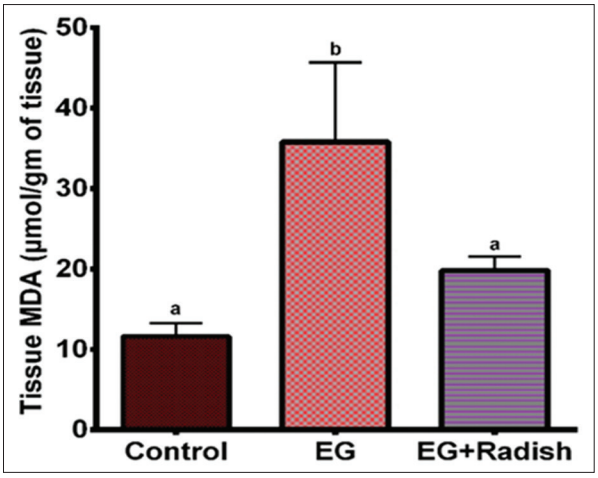

Figure 4: Level of tissue malondialdehyde in control, model, and ethylene glycol +radish treated groups. Different letters on bars mean significant change and the same letters means no significant change

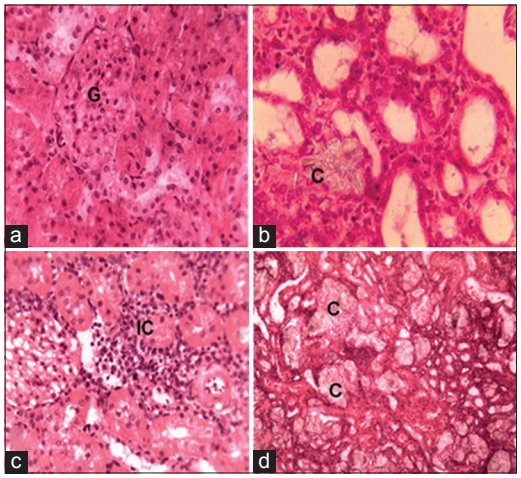

Figure 5: Kidney sections of control group (a) show normal appearance(G): Glomerulus.×400. Hematoxylin and eosin (H&E). (B) Kidney sections of model group (b) show a lot of crystal deposition(C) ×400. H&E. Kidney section of radish treated group (c and d) shown inflammatory cells and crystal deposition (C) ×400. H&E

Creatinine reacts with picrate to form a colored complex in alkaline solution. Absorbency of samples was read at 500 *nm*.

Determination of Kidney Tissue Malondialdehyde (MDA)

The right kidney of each rat was washed in ice-cold normal saline solution then homogenized in 20 mM phosphate buffer (pH = 7.4; tissue/buffer ratio, 1/10 *w/v*) by handheld glass homogenizer (Chowdhury *et al*., 2013). Homogenates were centrifuged at 4000 *g* at 4°C for 10 *min* (Beckman J2-21). The supernatants were collected and stored at −8°C until assayed. MDA level was estimated according to method of Kartha and Krishnamurthy (Kartha and Krishnamurthy, 1978). 1 *ml* of tissue homogenate was added to 20% trichloroacetic acid. After centrifugation for 10 *min*, 2 *ml* of supernatant was taken in a test tube and 2 *ml* of 0.7% thiobarbituric acid was added to each tube and kept it in the boiling water bath for 20 *min*. The development of pink color was measured at 535 *nm* and MDA concentration was calculated by the following equation:

$$\text{MDA nmol/g of tissue} = \text{Absorbance at 535 nm} \times D / (L \times Eo)$$

L: Lightpath (1 cm)

E o: Extinction coefficient 1.56×105 M−1.cm−1

D: Dilution factor.

Light Microscopy

Kidneys were removed from the anesthetized animals, immediately fixed in Bouin's solution for 24 *h* followed by dehydration using a series of ethanol in ascending concentrations (50%, 70%, 95%, and 100%), then immersed in xylene for clearing process, infiltrated with paraffin wax, and embedded in paraffin wax. 5 μm thick paraffin sections were obtained using rotary microtome (Bright, MIC) and stained by hematoxylin and eosin. The specimens were examined and photographed under light microscope (digital binocular compound microscope ×40–×2000, built-in 3MP USB camera).

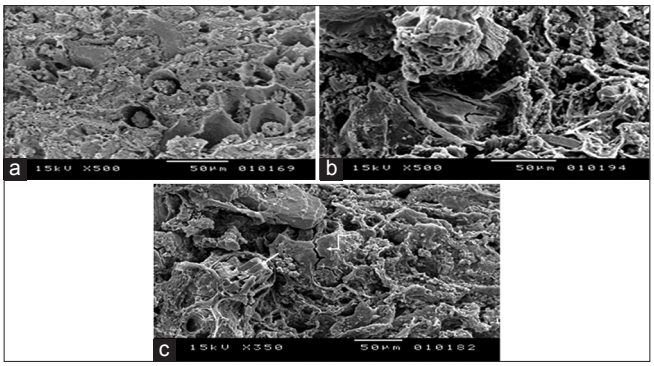

Figure 6: Scanning electron microscopy of kidney (a) control group showing normal appearance (b) ethylene glycol (EG)-treated group showing crystal deposition (white arrow) and calcified tissue (blue arrow). (c) EG + radish treated group showing more crystal deposition (white arrow) and calcified tissue (elbow arrow)

Electron Microscopy

Scanning electron microscopy was done at the University of Niece, France. Kidneys were fixed in 2.5% glutaraldehyde in 0.1M cacodylate buffer *pH* 7.2–7.4. After washing dehydrated in ethanol (50%, 70%, 85%, 100%, and 100%), the samples were put in desiccator for air drying; after mounting, they were coated by coater machine with gold and then examined by scanning electron microscope (SEM).

Statistical Analysis

Data entry was done by Microsoft excel 2010 and analyzed statistically by one-way analysis of variance using GraphPad Prism *version* 6.01 and SPSS *version* 20 and data expressed as mean ± standard error. Newman–Keuls tests were chosen as *post hoc* tests.

RESULTS AND DISCUSSION

As shown in Figure 1, both the left and right kidneys weight in model and radish-treated group increased significantly when compared with control group. Although many cells may degenerate in response to EG toxicity or to the formation and presence of crystals in the kidney, the increase in kidney weight in EG and EG-treated plant juice was expected at least due to the presence of renal crystals.[18] Fluid intake [Figure 2a] in EG and radish-treated groups elevated in compare of control group. Increasing fluid intake in EG-induced group maybe return to sweet taste of EG[19] or nephron damage that could not reabsorb glomerular filtrate again.

As illustrated in Figure 2b, urine flow in model group increased significantly when compared to control group; however, there is no statistical difference between model and radish-treated group. With respect of serum creatinine, Figure 3a shows significant raising in EG and radish-treated groups when compared to control group, while there is no statistical difference between both EG-treated groups. The elevation of serum creatinine may be due to renal tubules obstructions.[20] Figure 3b revealed that there was no statistical difference of serum urea between control and model group and between model and radish treated group but serum urea elevated in radish group when compared to control group. Increasing of serum urea is related to the level of nephron injury[21] and since more crystals were deposited in the kidney of EG plus radish group leading to more kidney damage, this may explain the higher serum urea noticed in this group in comparison to the control group. Furthermore, urea is by product of protein metabolism and this rising in serum urea may return to the fact that radish itself contain total protein of 6.5%.[22] As shown in Figure 4, the level of MDA in model group raised when compared with control group while there was no significant difference between control and radish treated group. MDA is by product of lipid peroxidation and is used as marker for this process and oxidative stress induced by EG through elevating MDA level confirmed previous findings.[23,24] Under hyperoxaluria, oxalate reacts with polyunsaturated fatty acid in cell membrane[25] and make damage. Calcium oxalate which induced oxidative stress can help crystal attachment to epithelial cells of nephrons (cell-crystal attachment).[26] Radish juice succeeded in lowering the MDA level compared with EG group and it may be due to the antioxidant contents of radish since it has been found to contain phenolic compounds and ascorbic acid.[27]

Paraffin sections of kidney belong to control group (Figure 5a) show normal and healthy appearance. Kidney sections of model group have shown crystal deposition and dilated tubules [Figure 5b] that is match with finding of the previous study.[28] Inflammatory cells (IC) accumulation also observed that is maybe due to ability of calcium oxalate to triggering immune system.[29] Oxalate, the major stone-forming constituent, has been reported to induce lipid peroxidation and causes tissue damage by reacting with polyunsaturated fatty acids in cell membrane.[23] On the other hand, some investigations say that lipid peroxidation is not the underlying cause of renal injury in hyperoxaluric rats.[30] Paraffin section through the kidney of EG + radish juice treated rats has shown more quantity of crystal deposited in lumen of kidney tubules [Figure 5d] and a lot of inflammatory leucocytes were observed in kidney tissues [Figure 5c]. Radish has been found to contain four major oxalic, malic, malonic, and erythroic acid.[31] Oxalic acid itself is one of the promoters of renal stone formation. Despite promoter activity of oxalic acid, total acidity of these four acids may decrease urine pH and makes a favor environment for calcium oxalate crystals.

Scanning electron microscopy showed the three-dimensional image of crystals more clearly in comparison to other techniques. The kidneys in the control group were appeared with normal and healthy structure having no crystals [Figure 6a]. Clear large

crystals were revealed in the kidney tissue of EG-treated rats [Figure 6b]. SEM of rat kidneys belong to EG + radish juice treated group showed crystals deposition in wide distribution and in the most area, the tissue appeared calcified [Figure 6c].

CONCLUSION

Radish treated group has shown more crystal deposition, damage, and IC in compare to model; however, radish juice decreased MDA level significantly. Radish juice could not improve the state of experimental rats in kidney weight, renal function tests, and fluid intake.

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Research Article

Omega-3 Oil Ameliorate Histological and Ultrastructural Alterations Induced by Cadmium Chloride in Rats Testis

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ABSTRACT

Cadmium (Cd) is considered to be one of the major environmental pollutants which have potential threat to human health. Reports of declining male fertility have renewed interest in the role of environmental and occupational exposures in the etiology of human infertility. Cd exposure led to obvious degenerative changes in testicular tissue. This study was performed to investigate the Cd-induced structural effects on the testes and to evaluate the possible protective effect of omega-3 oil in adult albino rats. Thirty adult male rats were used in the present work, divided randomly into five groups, six rats in each group; the first group was considered as a control group and left without treatment except the standard rat chow and tap water. The second group was given 40 mg/L of $CdCl_2$ in drinking water while the third group was given 60 mg/L of $CdCl_2$ in drinking water. The fourth group was given 40 mg/L of $CdCl_2$ in drinking water plus omega-3 oil (4 g/kg diet), while the fifth group was given 60 mg/L of $CdCl_2$ in drinking water plus omega-3 oil (4 g/kg diet), the Cd-treated rats showed dose-dependent histological and ultrastructural alterations which have been ameliorated after exposure to omega-3 oil. The present investigation concluded that omega-3 played a protective role against Cd-induced histopathological changes in rat testis.

Keywords: Cadmium chloride, omega-3, testis, ultrastructural

INTRODUCTION

Cadmium (Cd) is a widespread toxic environmental and industrial pollutant. It is listed by the U.S. Environmental Protection Agency as one of 126 priority pollutants.[1] A great attention has been paid to Cd pollution by environmentalists due to its toxicity to plants, animals, humans, and even microorganisms.[2,3] Cd concentration in the environment has increased as a consequence of anthropogenic activity.[4] It is released into the environment by mining and smelting activities, atmospheric deposition from metallurgical industries, incineration of plastics and batteries, land application of sewage sludge, and burning of fossil fuels.[5] It concentrates along with the food chain and once absorbed irreversibly accumulates in the human body, particularly in kidneys, bones, respiratory tract, and other vital organs such as the lungs and the liver.[6]

Cd found to induce testotoxicity[7,8] through the production of reactive oxygen species and inhibition of antioxidant enzymes.[9,10] The protective role of omega-3 oil against the toxicity of several toxicants, some of them were minerals such as arsenic,[11] has been studied. However, as far searched, no such studies on the protective effect of omega-3 oil against Cd toxicity were found.

Cd is considered as one of the most toxic transition metal elements that can cause severe damage to testis,[7,8] induced severe necrosis followed chronic degeneration in rat testes, and also induced sex accessory gland atrophy and reduced circulating testosterone through reducing Leydig cell function.[12,13] However, its effectiveness dependent on the dose of exposed Cd.[14] Alhazza[15] showed that administration of 2.5 mg/kg S.C. $CdCl_2$ to male rats caused changes in testosterone and gonadotropic hormones. Cd accumulated in testes in response to time and dose increased oxidative stress enzymes, endocrine disruption, and altered apoptosis.[16] Cd given parenterally to pregnant laboratory animals induced a variety of adverse reproductive change, decreased litter size, resorption, fetal death, growth retardation, and congenital malformations in offsprings of exposed animals.[17] In female rats subcutaneously injection on the 6th day of gestation with different doses of $CdCl_2$ caused an increase in testes diameters

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of seminiferous tubules, a progressive sloughing of germ cells and vacuolization of Sertoli cells and Leydig cells hyperplasia in the pups were noted.[18]

Ercolani *et al*.[19] demonstrated a statistically significant decreased spermatogenesis and testosterone levels as well as discrete changes in Ca^{+2} homeostasis gene regulation in the presence of increasing levels of Cd in a rat model. Acute Cd exposure resulted in hemorrhagic injury to the testis, although some strains of animals were resistant to this effect.[20] Cd severely damaged the seminiferous tubules and caused degeneration and disintegration of spermatogenic cells. Leydig cells were also lost after Cd treatment.[21]

The testes of male albino rats intoxicated with $CdCl_2$ showed edematous swelling, congestion, hemorrhage, focal to multifocal white pale depressed areas of ischemic necrotic patches, increased oxidative stress, and histopathological and biochemical effects.[22,23] Several studies focusing on Cd-related changes in testicular histopathology have implicated testicular blood vessel damage followed by the degeneration of spermatopoietic epithelial, as the main cause of Cd toxicity.[24-26] Fouad *et al*.[27] found testicular damage induced by a single intraperitoneally injection of $CdCl_2$ (2 mg/kg), the mechanism by which Cd affected testis was oxidative stress and inflammation. Intraperitoneal injections of $CdCl_2$ caused a marked and prolonged reduction of spermatogenesis, accompanied by increased permeability of the blood–testis barrier.[28]

Cd has been found to cause obvious adverse effects on the proliferation of piglet Sertoli cells and caused their DNA damage, cell apoptosis, and aberrant morphology.[29] Treated rats with $CdCl_2$ at a single dose of 2 mg/kg B.W showed various degree of testicular degenerations such as degeneration and necrosis of germinal cells and irregularity and atrophy of seminiferous tubules.[30] Krichah *et al*.[31] and Toman *et al*.[32] showed that exposure to Cd also induced a pronounced alteration of spermatogenic process with dramatically reductions of spermatozoa produced in the lumen of the seminiferous tubules sections and a decrease of the intratubular tissue volume, vacuolation in the epithelium, and necrosis were appeared. De Souza Predes *et al*.[33] found that Cd significantly reduced the seminiferous tubules diameter and decreased volume density of seminiferous tubules. The tubules lumen was filled with degenerated germ cells and multinucleated spermatid aggregates, vacuolization of the seminiferous epithelium was also observed. By scanning electron microscopy (SEM) examination, the absence of spermatozoa and height diminishing of seminiferous epithelium were appeared.

A serious decrease in the level of testosterone, a significant elevation in serotonin, increased oxidative stress in testicular tissue, poor semen quality (sperm count, sperm motility, and sperm viability), and histopathological alterations in the testis of Cd-treated rats have been declared by several studies.[34-36] Cd exposure (1.1 μg/g of diet) to the male rats caused increased plasma testosterone levels significantly, while epididymal sperm concentration significantly decreased in contaminated rats as compared to correspondent controls.[37,38]

Zararsiz *et al*.[39] concluded that omega-3 FAs had favorable effects in rat testis tissue by preventing oxidative damage and increasing the level of testosterone. Omega-3 FAs have effective protection for testicular tissues against methotrexate-induced testicular toxicity male albino mice.[40]

Oral administration of omega-3 (2 ml/kg B.W) either before or after treatment with azathioprine was effective in decreasing the DNA fragmentation and total sperm abnormalities and significantly increasing the sperm count.[41] The data of Uygur *et al*.[42] suggested that fish omega-3 FAs pre-treatment may be beneficial for spermatogenesis following acute doxorubicin (DOX)-induced testicular damage by decreasing germ cell apoptosis and oxidative stress.

MATERIALS AND METHODS

Thirty adult albino male rats (*Rattus norvegicus*) (200–250 g and 8 weeks old) were used in the present study. They were bred and housed in plastic cages (56 cm × 39 cm ×19 cm), bedded with wooden chips in the animal house of Biology Department/College of Science/University of Salahaddin, Erbil. The animals were kept under standard laboratory conditions 12 h light:12 h dark with controlled temperature of 22 ± 2°C. The rats were given standard laboratory chow containing 0.5% NaCl, 22% protein, and 4–6% dietary fat[43] and drinking water *ad libitum*.

Experimental Design

The rats were divided randomly into five groups (each contained six rats), the first group serves as a control was the rats given standard rat chow and tap water, the second group was treated group given (40 mg/L $CdCl_2$ in drinking water), the third group given $CdCl_2$ (60 mg/L in drinking water), the rats in the fourth group were received $CdCl_2$ (40 mg/L in drinking water) + omega-3 oil (4 g/kg diet), the omega-3 oil purchased from local pharmacy, and the last group received $CdCl_2$ (60 mg/L in drinking water) + omega-3 oil (4 g/kg diet), the experiment period for all groups was 30 days.

For studying the effect of $CdCl_2$ and/or omega-3 oil, all animals were anesthetized with ketamine hydrochloride (100 mg/kg B.W.) and sacrificed at the end of each experiment. Testes were taken from the rats and were cut into small pieces (<0.5 cm^3 in thickness) and kept in the fixative.

Histological Preparations

Light microscopy (paraffin method)

Samples of the testes were directly fixed in Bouin's fluid for 24 h and then processed for paraffin method by dehydrating through ascending concentrations of ethanol (50%, 70%, 95%, and 100%), cleared in xylene, infiltrated in paraffin wax, and finally embedded in paraffin wax. Sections were cut at 4 μm thickness with a rotary microtome (Hunting Don, Bright. UK). The sections were stained by hematoxylin and eosin method.[44,45]

Electron microscopy

Tissue samples (1 mm^3) were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2–7.4 for 24 h, post-fixed in 1% osmium tetroxide for 1 h, dehydrated through a graded series of acetone (50%, 70%, 95%, and 100%), cleared in propylene oxide for 15 min (twice), infiltrated with propylene oxide plus

Araldite mixture (1:1) for 12 h, and then embedded in resin medium (Araldite CY 212: 10 g; Hardener 10 g; Accelerator DMP 30: 0.5 g; and di-n-butylphthalate plasticizer: 0.6 g). All these chemicals were obtained from Fluka and Bucha. Polymerization was accomplished in an oven at 60°C for 48 h.[46]

The blocks were cut by ultramicrotome (Reichert-Jung) into 0.5–1 μ thickness and stained by 1% toluidine blue in 1% borax for light microscopy. For transmission electron microscopy (TEM), 600–900A° sections were obtained and stained by 3% uranyl acetate and lead citrate.[47] These ultrathin sections were examined by (SHARP) TEM in Gazi University, Faculty of Medicine, Ankara, Turkey and (LEICA/Em FC6, CM12 Philips) TEM in UKM University, Science and Technology Faculty, Malaysia.

For SEM, testes were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2–7.4 for 24 h. After washing by the same buffer, they post-fixed in 1% osmium tetroxide for 1 h, dehydrated in ethanol (50%, 70%, 85%, 100%, and 100%). The samples were put in desiccator for air drying. After mounting, they were coated with gold by coating system (E5200 AUTO SPUTTER COATER),[48] then examined by (ZEISS, super A, 557P) SEM in UKM University, Science and Technology Faculty, Malaysia.

RESULTS

Both doses of $CdCl_2$ (40 and 60 mg/L) caused many histological and ultrastructural alterations in the testis, on the other hand, omega-3 oil was very useful in minimizing most of the $CdCl_2$ toxic effects.

As shown in Figure 1, a healthy histological structure of rat testis having a germinal epithelium undergoing cell division and well-formed spermatids is seen. Administration of low dose of $CdCl_2$ to the male rats has caused a histological damage to the germinal cells and also affected the spermatids production [Figure 2a and b]. The affected germinal cells appeared highly vacuolated and enlarged spermatogonia and showed Cd particles deposition [Figure 2c and d]. Further, damages due to $CdCl_2$ effect on the other cells of the interstitial space such as Leydig cells were noticed. Such effect included condensed and crescent-shaped nuclei which looked like characteristic apoptotic feature in addition to the appearance of a large number of vacuoles [Figure 2e and f]. These changes have been confirmed by the electron micrographs [Figure 3a and b], in which ultrastructural alterations were clearly appeared such as highly spermatids degeneration with shrunken nuclei, high number of vacuolation in all germinal

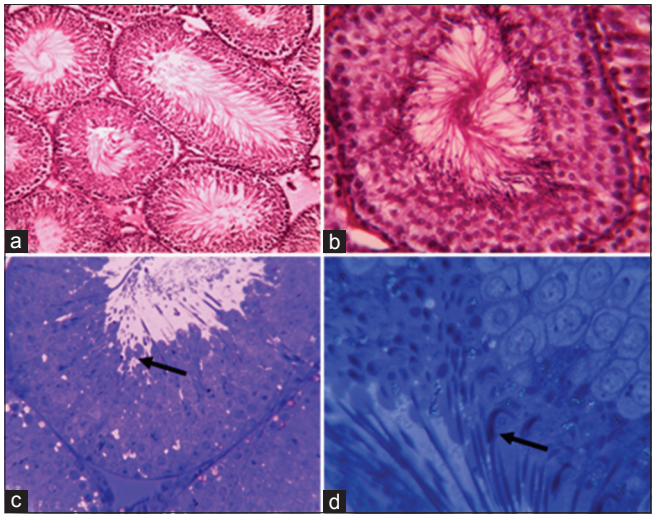

Figure 1. Sections through the testes of control rats, A&B) Paraffin sections showing normal histological structure of seminiferous tubules with a lot of spermatids in the lumen of the tubules, 100x and 400x respectively, H&E, C&D) Plastic sections showing normal germinal layer cells and the newly formed spermatids (arrow), 400x and 1000x respectively, toluidine blue.

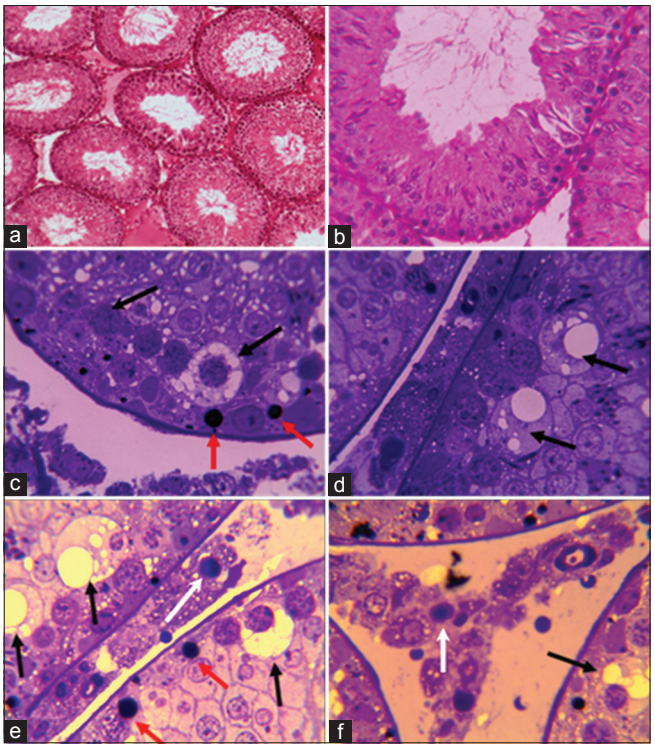

Figure 2. Sections through the testes of the low dose of CdCl2 treated rats, A and B) Low power of seminiferous tubules showing slightly empty lumen of seminiferous tubules 100x and 400x respectively, H&E, C-F) Different vacuolated spermatocytes (black arrows), degenerated Leydig cells (white arrow), precipitated Cd particles (red arrows) 1000x, toluidine blue.

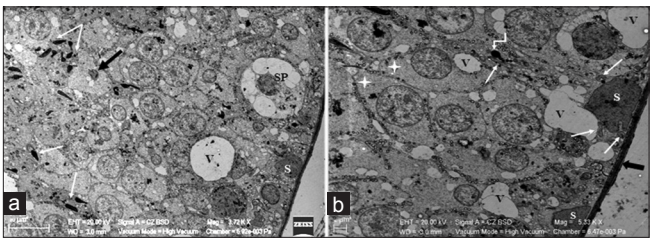

Figure 3. Electron micrographs through the testes of the low dose CdCl2 treated rats showing, A) Spermatid degeneration (white arrow), shrinkage of spermatocyte nucleus (black arrow), highly vacuolated spermatogonia (SP), vacuole (V), Sertoli cells (S). B) Thickining of basal lamina (black arrow), large and ✧small vacuoles in the germinal cells (V), shrunken Sertoli cells (S) and Cd particles (), notice the Golgi complexes (), different shape and size of mitochondria (white arrow), cup shape of spermatids head()

epithelium, especially the spermatogonia, thickening of the basal lamina, variation in the shape and size of mitochondria, and deposition of Cd particles.

Higher magnification of the later electron micrographs showed very large vacuoles with high deposition of Cd particles, lysis of mitochondrial membrane, high electron-dense sperm, and dilation of endoplasmic reticulum [Figure 4a and b]. Similar to the low dose, the high dose of CdCl2 caused similar damages to the germinal layer, in which no spermatids were seen in the lumen of the seminiferous tubules [Figure 5]. More alteration details were observed by electron microscopic images in the high-dose $CdCl_2$-treated rats which included approximately less spermatids production with defect heads and tails [Figure 6a], vacuolated and degenerated Leydig cells [Figure 6b].

A clear protective role of omega-3 oil was detected in the testis of $CdCl_2$-treated rats in both doses, in which an approximately normal histological structure of the seminiferous tubules was seen [Figure 7].

The scanning electron microscopic images have confirmed the histological and ultrastructural results regarding the testicular effect of $CdCl_2$ and the protective role of omega-3 oil [Figures 8 and 9]. As revealed by these figures, the cells of the germinal layer of the control group appeared filled the lumen of the seminiferous tubules with a large number of well-formed spermatozoa [Figure 8]. The little numbers of sperms and the presence of vacuolation in the germinal layer were clearly appeared in the testis of $CdCl_2$-treated group [Figure 9a].

The protective role of omega-3 oil against the $CdCl_2$ toxicity in the testis of male rat was clearly seen by the scanning electron microscopic image, in which a lot of spermatozoa were reappeared in the lumen of the seminiferous tubules [Figure 9b].

DISCUSSION

Spermatogenesis is a complex multitemporal process, including proliferation and differentiation of spermatogonia, meiosis, and spermiogenesis. In this process, any of the

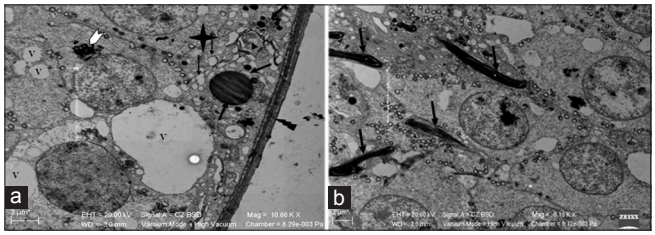

Figure 4: Electron micrographs through the testes of the low dose of $CdCl_2$-treated rats, (a) high deposition of Cd particles (thick arrow), thickening of basal lamina (⊲), lysis in mitochondrial membrane (head of arrow), spermatocyte with high electron density (➘), vacuolation in primary spermatocytes (V), and dilated smooth endoplasmic reticulum (thin arrow). (b) Degeneration of spermatid heads (black arrows) with vacuolation and lysed mitochondria (white arrow)

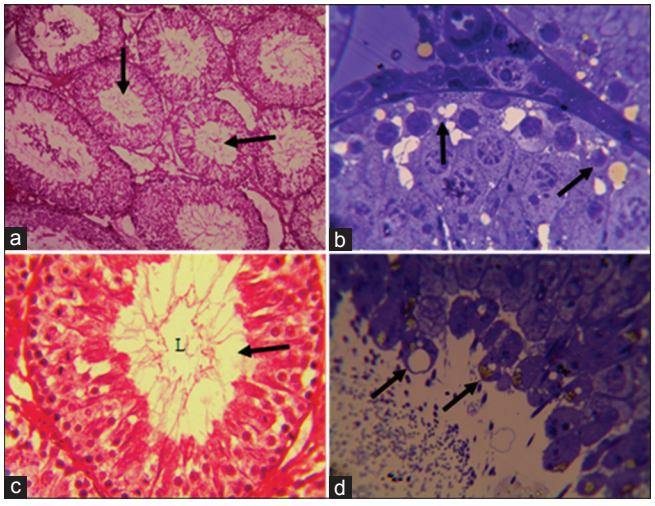

Figure 5: Sections through the testes of the high dose of $CdCl_2$-treated rats, (a) paraffin section through a number of seminiferous tubules showing empty lumen (arrows), ×100, (b) plastic section through a part of the germinal epithelium showing vacuolation of the cells (arrows), ×1000, (c) large lumen of seminiferous tubule and very little spermatids, ×400, (d) vacuolated spermatocytes with yellow lipid droplets (arrows) near the lumen (L), (arrows), ×1000

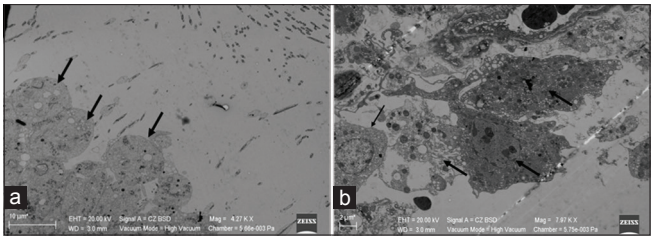

Figure 6: Electron micrographs through the testes of the high dose of $CdCl_2$-treated rats showing, (a) vacuolated spermatocytes (arrows), (b) healthy (thin arrow) and degenerated Leydig cells (thick arrow) showing a phagocytosis mechanism

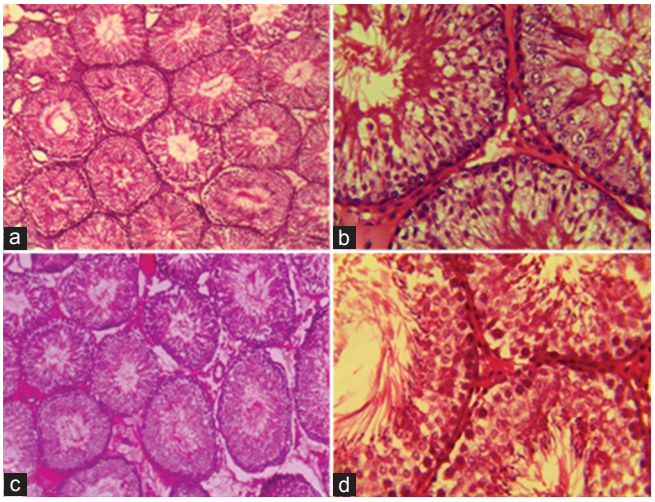

Figure 7: Paraffin sections through the testes of $CdCl_2$ plus omega-3 treated rats, (a) low dose of $CdCl_2$ plus omega-3 showing approximately normal structure of seminiferous with high quantity of spermatids in the lumen of the tubules, ×100, (b) higher magnification of the seminiferous tubules in the latter section, ×400, (c) high dose of $CdCl_2$ plus omega-3 treated rat group showing nearly normal seminiferous tubules, ×100, (d) same latter group showing number of spermatids and the healthy germinal cells undergoing cellular division, ×400, hematoxylin and eosin

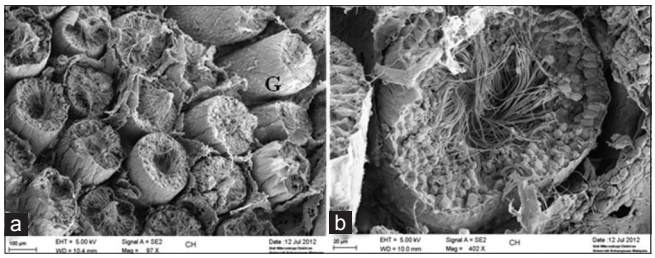

Figure 8: Scanning electron microscopy image through the testes of the rats in the control group showing normal seminiferous tubules containing high number of sperms (S), germinal epithelium (G), (a) low power, (b) high magnification

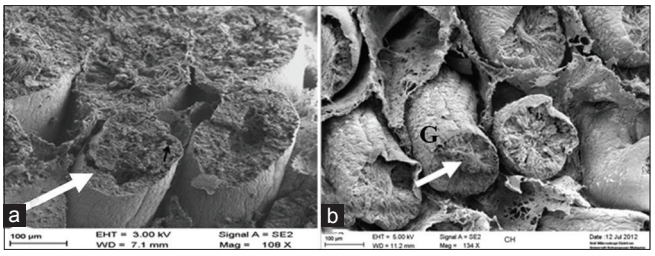

Figure 9: Scanning electron microscopy images of seminiferous tubules of (a) low dose of $CdCl_2$-treated rat testis showing little sperms in the lumen of the tubules and the formation of vacuoles in the germinal epithelial cells (arrows). (b) Low dose of $CdCl_2$ plus omega-3 treated group showing a lot of sperms in the lumen of seminiferous tubules (arrows)

affected areas are likely to cause spermatogenesis impairment and even infertility.[49,50]

Heavy metals could adversely affect the male reproductive system, either by causing hypothalamic-pituitary axis disruption or by directly affecting spermatogenesis, resulting in impairs semen quality.[51]

The present results showed different histological and ultrastructural alteration in rat testis, which included decrease in germinal epithelium thickness, slightly empty seminiferous tubule, and highly vacuolation features. These findings are in accordance with the previous observations.[30,52-53] Several studies focusing on Cd-related changes in testicular histopathology have implicated testicular blood vessel damage followed by the degeneration of spermatopoietic epithelial, as the main cause of Cd toxicity.[26,54]

Other studies on experimental animals have brought the evidence that oxidative stress is implicated in the toxicity of Cd.[55,56] The treatment with Cd (0.4 mg/kg B.W.) intraperitoneally caused significantly increased lipid peroxidation in rat tissues, enhancing peroxidation of membrane lipids altering the antioxidant system of the cells, which may cause injury to cellular components due to the interaction of metal ions with the cell organelles.[57]

Many ultrastructural defects on testes of rat testes were observed in the present study such as spermatid degeneration, shrinkage of spermatocytes, deposition of Cd particles, thickening of basal lamina, large and small vacuoles in the spermatocytes, and shrinkage of Sertoli cells. The later result concerning the effect on Sertoli cells was observed recently by Luca *et al*.[58] Kamel *et al*.[36] revealed that the testes are greatly targeted to damage by Cd intoxication and increased oxidative stress resulted from Cd intoxication in testicular tissue of treated rats might be responsible in testicular damage and impairment of fertility.

It has been reported that the oxidative stress affects the sperm cell through interfering with the membrane fluidity which is the main factor for sperm motility and fusion with the oocyte.[59] The present results showed decrease in the thickness of the germinal epithelium of the seminiferous tubules the testicular toxicity of Cd appeared to be mediated by a rapid apoptotic process as revealed by the decrease in the density of sperms in the lumen of the seminiferous tubules of treated rats. These observations agree with other studies indicating the implication of apoptosis in the mechanism of cytotoxicity of Cd in testes.[31,60] The data of Minutoli *et al*.[61] confirmed that the i.p. administration of $CdCl_2$-induced significant seminiferous epithelium damages and increased TUNEL-positive peripheral germ cells were observed in the seminiferous tubules, indicating an involvement of spermatogonia and primary spermatocytes in apoptotic processes. The previous studies of acute Cd exposure have reported diminished testicular weight in relation to the Cd dosage.[62] These studies attributed to this effect to the necrotic and degenerative changes induced by Cd.[63] The study of Toman *et al*.[30] demonstrated that Cd caused decrease in the thickening in the wall of the seminiferous tubules, and this may be due to the degeneration of germ cell layers.

Increase in the levels of total reactive oxygen species and lipid peroxidation was shown in testicular tissue after Cd administration, these biochemical alterations are considered as one of the forerunners of pathological changes in testis after Cd administration, also reduced level of enzymatic antioxidants and increased production of free radicals severely affected the testicular biology in the presence of Cd.[8] Testicular effects of Cd may be due to Cd interference with zinc-protein complexes that control DNA transcription, subsequently leading to apoptosis.[64] Most of the criteria of apoptotic mode of cell death in the germinal epithelium revealed in other studies that dealt with Cd testotoxicity (such as condensation of nuclear chromatin and degeneration of cytoplasmic organelles, especially the collapse of mitochondria)[50] were detected in the present work. Elmallah *et al*.[65] have been recently reported that oxidative stress and elevated levels of reactive oxygen species (ROS) are the main cause of cellular damage as a result of Cd exposure.

As revealed by the current investigation, omega-3 FO was succeeded in protecting the testis against most histological and ultrastructural changes caused by Cd. The most expected reason for this protection may be through oxidative/antioxidative mechanism. Similarly, Uygur *et al*.[42] suggested that fish omega-3 FAs pre-treatment may be beneficial for spermatogenesis following acute DOX-induced testicular damage by decreasing germ cell apoptosis and oxidative stress. Recently, Cd was found to decrease serum testosterone in rats.[7] Furthermore, omega-3 was found to elevate the level of testosterone in rats.[39] Such elevation of testosterone may the reason behind the protection of germ cells against the degenerative effect of Cd which has been detected in the present work since this hormone has been shown to regulate apoptosis in adult human somniferous tubules *in vitro*.[66]

CONCLUSION

The present investigation concluded that omega-3 oil could play a protective role against cadmium induced testicular toxicity due to the antioxidant power of the oil.

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Cihan University-Erbil Scientific Journal (CUESJ)

Research Article

Bromodomain Inhibitor JQ1 as a Candidate Therapeutic Agent in Malignant Pleural Mesothelioma

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ABSTRACT

Malignant pleural mesothelioma (MPM) is a rare tumor that develops from the mesothelial linings of the pleural, pericardial, and peritoneal cavities. The actual risk factor for developing the disease is exposure to asbestos in workplace. Bromodomain and extraterminal (BET) domain proteins are epigenetic signaling agents that associate with acetylated histones and expedite the transcription of target genes. This study investigates whether the small molecule BET protein inhibitor JQ1 specifically may be an effective therapy for MPM. Reverse transcriptase polymerase chain reaction methods reveal an inclusive change in gene expression implying that JQ1 is a potential inhibitor which targets the BET proteins. Our results report that JQ1 has tumor-suppressive effects as it significantly ceased cellular activity in MPM cell lines. We predict that JQ1 may be the promising therapy for pleural mesothelioma cancers.

Keywords: Bromodomain and extraterminal domain, JQ1, malignant pleural mesothelioma

INTRODUCTION

Malignant pleural mesothelioma (MPM) is an infrequent type of cancer that affects the pleura (the external lining of the lungs and the internal lining of the chest cavity). MPM includes three main types determined by their appearance under the microscope. MPM includes three main types determined by their appearance under the microscope as: Epithelial MPM which is the most popular form, Sarcomatoid MPM which is meant to be the most aggressive form and Biphasic MPM which mixes both Epithelial and Sarcomatoid MPMs. Pleural mesothelioma is commonly caused by the long-term exposure to asbestos fibers that induce pathogenic changes and eventually lead to cell injury, fibrosis, and possibly cancer. It has been also proposed that aside from asbestos, there are other factors which have a role in the tumor pathogenesis, such as SV40 virus infection and genetic susceptibility.[1]

MPM incidence has been increasing over the world and is anticipated to grow even higher in the following 20 years due to asbestos huge spread exposure throughout the past 10 years or so.[2] It is rated that about 50–80% of pleural malignant mesothelioma in men and 20–30% in women arose in individuals who were exposed to asbestos.[3]

Diagnosis of this disease is pretty much difficult even for some specialists because the symptoms do not appear only after a longer period of exposure to asbestos. So far, the only approved treatment for MPM is the combined therapy of pemetrexed and cisplatin, although currently, there have been many clinical trials determining the efficacy of other drugs.

Bromodomain and extraterminal (BET) domain proteins play an essential role as epigenetic reader molecules that are associated with acetylated histones and aid in the transcription of target genes. Bromodomain is, therefore, an acetyl-lysine-binding motif of about 110 amino acids which are found in various transcription regulators and chromatin-modifying enzymes that are encoded by specific genes.[4] Research has shown that only members of the BET family (BRD2, BRD3, BRD4, and BRDT) may be prospective targets of cancer. These four BET proteins make up a subset of the larger family that consists of 46 proteins which contain 61 BRDs that have been determined in humans.[5]

BET inhibitors are getting to become of huge interest as a potential target due to the prospect that there might be biomarkers which will recognize the patients that may possibly benefit and respond to therapy. Those small molecule inhibitors conquer the acetyl-binding pockets of the bromodomains, leading in their detach from active chromatin and repression of downstream transcriptional targets.[6]

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JQ1 has started its journey in the research world as a novel drug in inhibiting the development of some specific cancers. JQ1 successfully blocks one of the well-known cancer-causing genes of the BET proteins which are BRD4, thereby preventing the early growth of some blood and lung cancers such as leukemia and multiple myeloma. JQ1 is a novel thieno-triazolo-1,4-diazepine having an attached bulky t-butyl ester functional group at carbon 6. This chemical compound is a vigorous, high affinity, selective BET inhibitor. JQ1 detaches BET proteins from chromatin by binding to the acetyl-lysine recognition pocket of BET. JQ1 has been used as a chemical probe to investigate the role of BET in the transcriptional regulation of oncogenesis.[7-9]

BET inhibitors are a new promising treatment for MPM. This study will focus on the effect of the BET inhibitor (JQ1) in MPM. We hypothesized that bromodomains of the BET family are expressed/overexpressed in MPM and hence investigate the effect of the compound JQ1 as an inhibitor of those BET. This appears to be the first study of its kind, as far as I know that reveals the role of inhibiting BET domain proteins with JQ1 in mesothelioma cancers.

MATERIALS AND METHODS

Cell Lines

In this project, two MPM cell lines were treated with the small molecule BET protein inhibitor and were assessed for antiproliferative activity as a response to that drug. The BET inhibitor used in this study was JQ1. The two cell lines used were REN and H226. A brief description of the cell lines as follows:

- REN is an epithelial mesothelioma cell line (fast growth rate)
- NCI-H226 is an epithelial squamous cell carcinoma; mesothelioma cell line (slow growth rate).

Cell Culture

Cell lines were typically grown and preserved at a fixed temperature of 37°C and a humidified environment of 5% CO_2 in a cell incubator. These cell lines were cultured in an appropriate cell culture media RPMI-1640 (Lonza, UK) which was supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, and 500 U/ml penicillin-streptomycin. Cell lines were regularly passaged in T75 cm^2 flasks (Nunc Flasks obtained from Fisher Scientific, Ireland) as following:

All cell culture procedures were done in laminar flow hoods under sterile conditions. Cell media discarded and cells were washed with 5 ml phosphate buffer solution. Cells were then trypsinized for 5 min at 37°C, and once detached from the flask, media were added to make up a final volume of 10 ml. Cells were pipetted many times and each 1 ml transfer to two new T75 flasks labeled with the cell line, passage number, and date. Further, 9 ml of media was added to each of these two flasks and cells were incubated at 37°C. In this way, cells were split at a ratio of 1:10.

Cell Subculture for Proliferation assays

Before drug treatment, cells were visualized under the microscope (Nikon Corp.) and if found to be of about 70–80% confluent, they were trypsinized with 1.5 ml trypsin-EDTA and 8.5 ml of media to make it up to a final volume of 10 ml. The cells were then counted using a bright-line hemocytometer **(**Sigma, USA**)** and seeded at 3×10^3 cells/well in 96-well plate. This was done in triplicate for both cell lines (REN and H226). The plated cells were kept in the incubator at 37°C.

Treatment of Cells

Once plated, cells were left to recover for 48 h. Subsequently, cells were treated with JQ1 BET domain inhibitor at the following concentrations (0.25 μM, 0.5 μM, 0.75 μM, 1 μM, 15 μM, 20 μM, and 25 μM) for 48 h. 4 μl of dimethyl sulfoxide (DMSO) was added to the control as JQ1 was dissolved in DMSO vehicle. A concentrated stock (18.6 mM) of JQ1 dissolved in DMSO was made and further four stock dilutions (1:25, 1:10, 1:5, and 1:2) were set up from that main concentrated stock. All plates were then incubated for 48 h at 37°C.

5-bromo-2-deoxyuridine (BrdU) Proliferation Assay

A BrdU assay was performed to measure the proliferative activity of the treated cells. Briefly, after cell treatment with JQ1 for 48 h, all media were removed from the triplicate 96-well plate, and subsequently, 10 μl BrdU labeling solution was added to each well. Plates were incubated at 37°C for 4 h followed by the removal of the labeling medium from the wells. 200 μl FixDenat was added to each well and plates were incubated at 25°C for 30 min followed by the removal of FixDenat solution thoroughly. 100 μl anti-BrdU POD solution was added to each well and plates were incubated again at 25°C for 90 min. The antibody was then removed and wells were rinsed 3 times with 200 μl/well washing solution. Finally, washing solution was removed and 100 μl/well substrate solution added to each well. Plates were left for 2–3 min and thence immediately 25 μl/well of 1 M H_2SO_4 was added and measurement of plates was carried out within 5 min. The activity of proliferative cells was measured by enzyme-linked immunosorbent assay at a wavelength of 450 nm using a standard plate reader.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Cell lines and tissue samples

Fresh-frozen mesothelial and benign pleural samples were collected following debulking surgery at Glenfield Hospital, Leicester, and were stored in The Leicestershire Mesothelioma Tissue Bank. Anonymized MPM specimens from 22 patients were transferred to St. James' Hospital, Dublin. Approval for the storage and use of these samples have been obtained from the St. James' Hospital/The Adelaide and Meath Hospital Research Ethics Committee. A panel of mesothelioma cell lines has been assembled and utilized for this project.

RNA isolation

Total RNA was isolated using TRI reagent® (MRC gene) protocol. Samples were mixed and incubated for 5 min at room temperature followed by centrifugation at 13,500 rpm for 5 min. The RNA pellet was left to air dry after the removal of the supernatant and finally resuspended in 30–50 μl of molecular grade water and stored at −80°C until further use.

RNA quantification

NanoDrop 1000 spectrophotometer (Thermo Scientific) was used to measure the purity of the isolated RNA. 1 μl of each RNA sample was loaded separately onto the NanoDrop and analyzed. The quantity of RNA per sample was measured in ng/μl. A 260/280 value of 2.0 indicates the purity of an RNA sample.

Complementary DNA (cDNA) synthesis

cDNA was synthesized using RevertAid RT according to the manufacturer's instructions (Fermentas). Samples were mixed gently, incubated for 90 min at 42°C followed by 10 min at 70°C heating to terminate the reaction. The resultant cDNA was then stored at −20°C until required.

RT-PCR

The cDNA which was synthesized previously was used to detect gene expression. For PCR, the following were mixed in a new tube to set up the PCR reaction: 10 μl (2× GoTaq® Green [Promega]), 2 μl (5 μM of each forward and reverse primers), 6 μl (molecular grade water), and 1 μl (cDNA). 1 μl molecular grade water was added in the place of cDNA for the negative control. PCR samples were loaded on a thermocycler. The PCR cycling conditions involved initial denaturation at 95°C for 5 min, followed by 30–35 cycles of denaturation at 95°C, annealing at the temperature 58°C, extension at 72°C, and a final elongation step at 72°C.

Gel electrophoresis

Agarose gel (2%) was used for loading all PCR products. Agarose (2%) was dissolved in Tris-acetate-EDTA (TAE) in the microwave for 3 min. 4 μl of ethidium bromide was added per 25 ml of agarose solution after being cooled and then poured into a gel tray. The gel was then allowed to set at room temperature for 10–15 min. 4 μl of 100 bp DNA ladder (Fermentas) was loaded onto the gel in parallel with the samples. 8 μl of each PCR product and 2 μl of the corresponding 18S housekeeping gene products were loaded as well. The samples were electrophoresed using an Electrophoresis Unit (Sigma) and 1× TAE was used as a running buffer. The gels were run at 80 volts for 30 min. The resultant bands were visualized and photographed under ultraviolet light using a biospectrum imaging system (Ultra Violet Products, Fusion FX, UK).

Statistical Analysis

Statistical analysis was carried out using two-tailed Student's *t*-test and one-way ANOVA. Data were graphed as mean ± standard error of mean using GraphPad Prism software. Densitometry analysis was performed using TINA software. Results were considered significant when the $P < 0.05$.

RESULTS

Screen of Panel of Mesothelioma Cell Lines for the Expression of BET Domain Proteins

A panel of 34 mesothelioma cell lines was screened for the expression of BRD2 and BRD4 (long and short transcript) variants. All these cell lines are MPMs, except for LP9, Met5A, NP1 and NP2, and P31 and P31CisR. Beta-actin was used as a positive control which is regularly expressed in all cell lines and a negative control was included also. BRD4 long transcript variant shows equal expression in all cell lines, but BRD4 short transcript shows a slightly lower expression in some of the cell lines [Figure 1]. BRD2 also shows an equal expression in all cell lines [Figure 2].

Screen of Primary MPM Patient Cells for the Expression of BET Domain Proteins

A panel of 34 primary mesothelioma cells obtained from patients was examined for the expression of BRD2 and BRD4 (long and short transcript) variants. These patient samples were divided into benign and three histological subtypes of MPM; epithelial, biphasic, and sarcomatoid. Densitometry analysis was performed to identify the differences in expression of the BRDs between benign and tumor tissue following normalization against the 18S rRNA expression for each sample, and a negative control was used in each PCR. BRD2 showed expression across samples [Figure 3a] and was significantly less expressed in the tumor samples compared to benign [Figure 3b and c].

BRD4 long transcript variant appeared to be expressed across the three histological subtype samples and less expressed in benign [Figure 4a] and following statistical analysis, BRD4 long transcript expression was significantly upregulated in

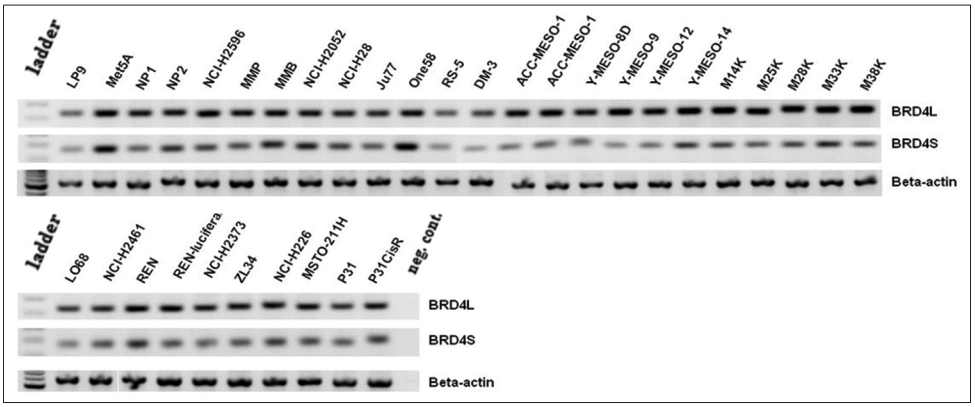

Figure 1: Expression of BRD4 long and short transcript variants in a panel of 34 mesothelioma cell lines. Beta-actin is a positive control. BRD4 long transcript is expressed uniformly in all cell lines and the expression of BRD4 short transcript is relatively less in some of the cell lines

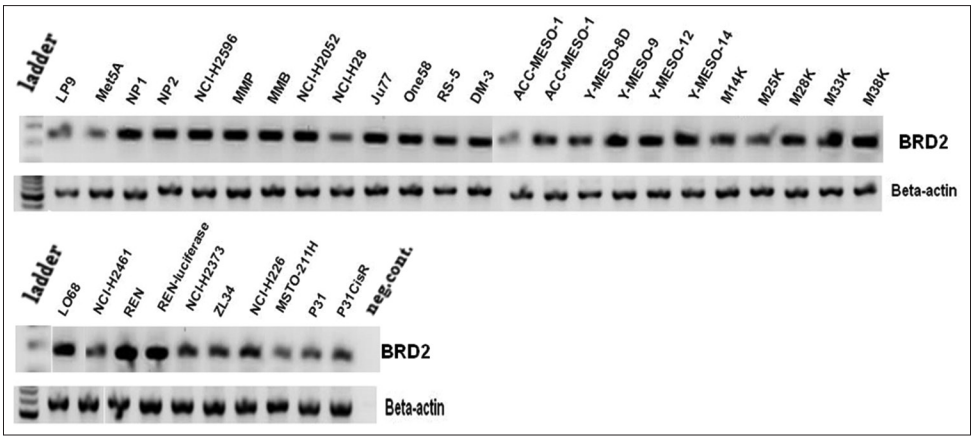

Figure 2: Expression of BRD2 in a panel of 34 mesothelioma cell lines. Beta-actin is a positive control. BRD2 is uniformly expressed in all cell lines

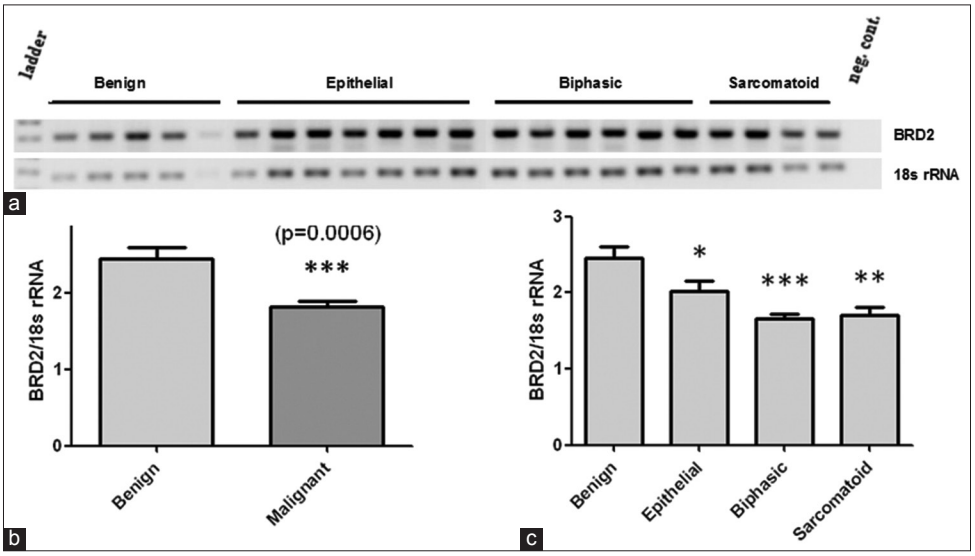

Figure 3: BRD2 expression levels. (a) Reverse transcriptase polymerase chain reaction for BRD2 levels in a panel of primary patient malignant pleural mesothelioma samples of different histological subtypes against 18S rRNA housekeeping gene. (b) BRD2 expression level in malignant patients is significantly less compared to benign (***$P < 0.001$, two-tailed t-test). (c) Levels of BRD2 expression in all three histological subtypes is significantly different compared to benign (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, one-way ANOVA using Dunnett's multiple comparisons test). Error bars represent mean ± standard error of mean

the tumor samples compared to benign [Figure 4b]. BRD4 long transcript expression levels were significantly higher in epithelial and biphasic subtypes compared to benign, while not significant in sarcomatoid [Figure 4c]. On contrary, the expression of BRD4 short transcript variant appears to be almost entirely absent in the benign samples [Figure 5a] and BRD4 short transcript expression was significantly upregulated in the tumor samples compared to benign [Figure 5b]. While not significant, a trend toward upregulation of BRD4 short transcript was observed in all three histological subtypes of MPM compared to benign [Figure 5c].

Antiproliferative Activity

The BrdU assay detects incorporated BrdU into the cellular DNA of actively proliferating cells. This assay was performed to measure changes in proliferating cells after 48 h following treatment with JQ1 drug. REN-treated cells appear to show a significant decrease in proliferation activity with 5, 10, 15, 20, and 25 μM drug concentrations, while NCI-H226 treated cells appear to show significant decline in proliferation with 0.25, 0.5, 0.75, 1, 15, and 25 μM drug concentrations [Figure 6]. The assay was accomplished in triplicate and the statistical analyses expressed as mean of treatments using one-way ANOVA and Dunnett's multiple comparison test.

DISCUSSION

MPM is a rare and aggressive form of cancer with limited treatment chances that have inconsiderable impact on survival improvement. Asbestos has been known as a non-mutagenic carcinogen that induces mesothelioma and in such case, cancer may be developed as an outcome of somatic epigenome dysregulation.[10] And so, aberrant epigenetic events have been spotted in MPM. Epigenome regulators include DNA methylation, histone modifications, chromatin remodeling, and regulation of non-coding RNAs. It is not clear why precisely epigenetic modifications are a notable feature of pleural mesothelioma,

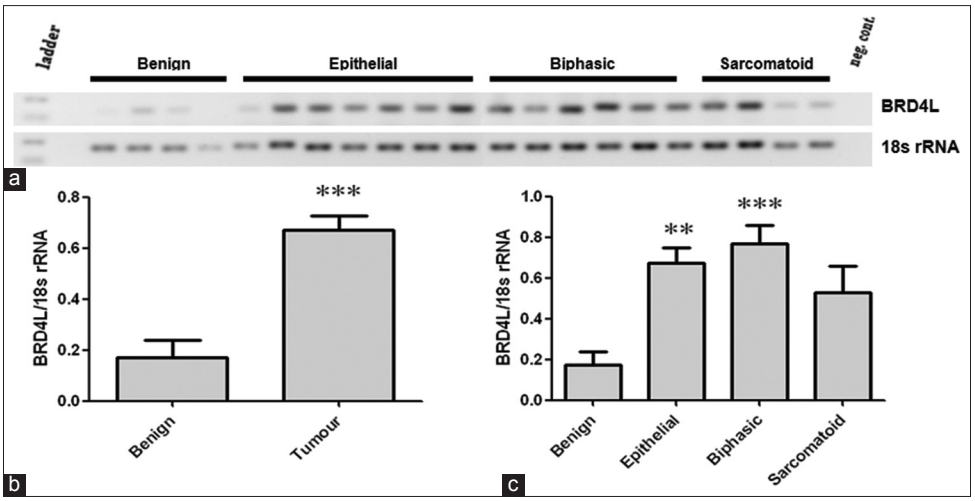

Figure 4: BRD4 long transcript expression levels. (a) Reverse transcriptase polymerase chain reaction for BRD4L levels in a panel of primary patient malignant pleural mesothelioma samples of different histological subtypes against 18S rRNA housekeeping gene. (b) BRD4L expression level in tumor patients is significantly different compared to benign (***$P < 0.001$, two-tailed *t*-test). (c) Levels of BRD4L expression epithelial and biphasic subtypes are significantly different compared to benign, while not significantly different in sarcomatoid subtype (**$P < 0.01$, ***$P < 0.001$, one-way ANOVA using Dunnett's multiple comparisons test). Error bars represent mean ± standard error of mean

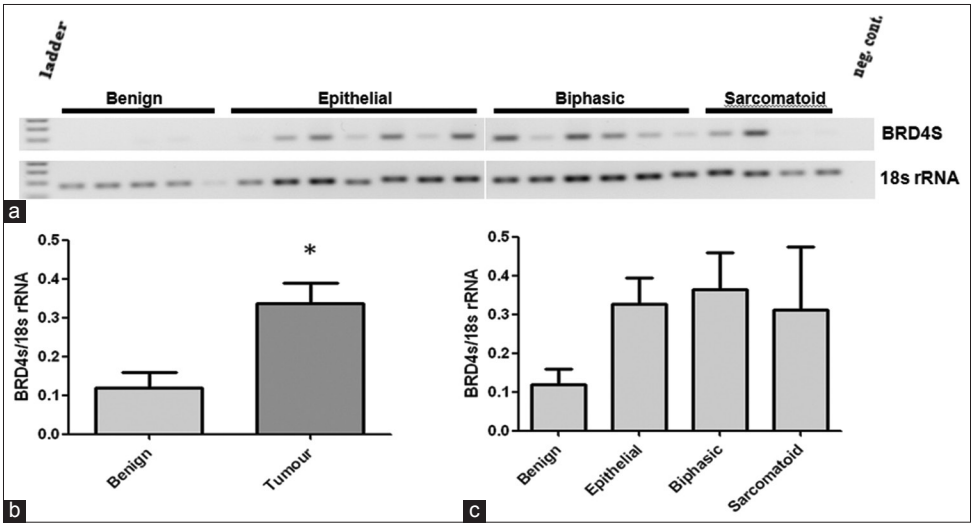

Figure 5: BRD4 short transcript expression levels. (a) Reverse transcriptase polymerase chain reaction for BRD4S levels in a panel of primary patient malignant pleural mesothelioma samples of different histological subtypes against 18S rRNA housekeeping gene. (b) BRD4S expression level in tumor patients is significantly different compared to benign (*$P < 0.05$, two-tailed *t*-test). (c) Levels of BRD4S expression in all three histological subtypes show no significance compared to benign (one-way ANOVA using Dunnett's multiple comparisons test). Error bars represent mean ± standard error of mean

but histone modifications have emerged as remarkable modifications in the carcinogenesis of MPM.[11] Aberrant regulation of histone modification plays a role in oncogenesis by affecting gene activity. The acetylation of histones is mediated by lysine acetyltransferases. Lysine acetylation is mainly recognized by bromodomains and tandem PHD domains.[12] BET proteins are readers of acetyl-lysine signs. In some cancer types, bromodomain-containing proteins which identify histone acetylation signs are mistargeted or overexpressed.[13]

This study focuses precisely on the role of two BET proteins, BRD2 and BRD4, in MPM and the effect of JQ1 compound as an inhibitor for BRDs. The expression of these BET proteins in MPM has been evaluated in a panel of mesothelioma cell lines and in primary MPM patient cells. A panel of cell lines [Figures 1 and 2] was screened to evaluate whether there are any prominent patterns of expression which need to be identified before screening of primary MPM patient cells. Slight changes in expression were noted. BRD4 short transcript variant is expressed weakly in some of the cell lines. Among the analyzed cell lines are a cisplatin resistance cell line. Cisplatin is a chemotherapeutic agent used in cancer treatment. A specific feature of MPM is that it is a cisplatin resistance tumor.[14] Although there were no remarkable variations in the expression level of any of the evaluated BRDs, additional MPM chemoresistant cell lines need to be included so that it would be a conclusive analysis.

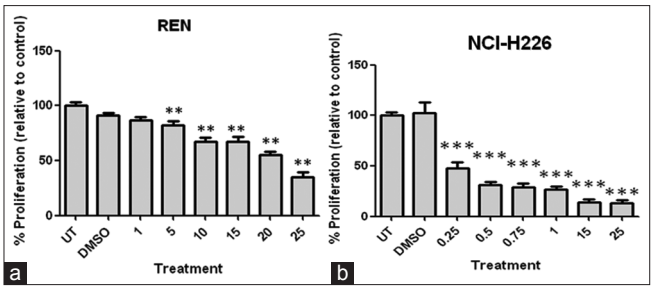

Figure 6: 5-bromo-2-deoxyuridine proliferation assay following 48 h treatment with various concentrations of JQ1. (a) REN cells show significant decline in cell proliferation at 5, 10, 15, 20, and 25 μM drug concentrations ($P < 0.01$, one-way ANOVA). (b) NCI-H226 cells show significant decline in cell proliferation at 0.25, 0.5, 0.75, 1, 15, and 25 μM drug concentrations ($P < 0.001$, one-way ANOVA)

Subsequently, a panel of primary MPM patient cells including benign, epithelioid, sarcomatoid, and biphasic histological subtypes was screened [Figures 3-5]. Expression of the three evaluated BRDs varies in malignant compared to benign cells. Expression of BRD2 was significantly different in malignant cells compared to benign cells. In addition, all three histological subtypes showed significantly lower levels of BRD2 expression compared to benign even though BRD2 was expressed in benign. On contrary, BRD4 long transcript expression levels are significantly higher in malignant cells compared to benign. In consistent with the results of BRD4 long transcript expression levels, the expression of BRD4 short transcript confirmed significantly elevated levels in malignant cells compared to benign, and this indicates that BRD4 long and short transcripts are overexpressed in malignant cell. Our findings have proven that BRD2 is downregulated in MPM tumors, while BRD4 long and short transcripts are significantly upregulated in MPM tumors. Moreover, since BRD4 is the main target for JQ1, consequently, this makes targeting MPM with JQ1 and excellent strategy.

To further support our findings, the antiproliferative activity of JQ1 was evaluated *in vitro* using two specific MPM cell lines (REN and NCI-H226). These two cell lines were chosen according to their common use in human tumor xenografts, as they perform well when xenograft in mouse models and so, the evaluation of JQ1 drug can be smoothly carried out in such *in vivo* models. JQ1 is a BET inhibitor that has been selected for this study. Scientists have been competing to develop novel BET protein inhibitors. A recently developed dihydroquinazoline-2-one inhibitor has been found to target BET.[15] BrdU assay was the first move in analyzing the proliferative activity of JQ1 compound. This assay detects BrdU incorporated into the newly synthesized DNA of actively proliferating cells. BrdU assay was chosen to measure the proliferating activity of cells. REN and NCI-H226 cells were treated with a range of JQ1 concentrations [Figure 6]. Response of NCI-H226 cells to JQ1 was clearly observed, with a significant decrease in cell proliferation starting from 0.25 μM up to 25 μm concentrations compared to untreated cells. While REN cells showed a significant decrease in proliferative activity with concentrations from 5 μM up to 25 μM. DMSO was used as a control to ensure that any change in proliferative activity was caused by JQ1 and not DMSO. DMSO induced no change in the cellular activity of both cell lines and so, noted results are accurate. JQ1 inhibits the proliferative activity of REN and NCI-H226 cells with concentrations of 5 μM and 0.25 μM, respectively. JQ1 has shown to have antiproliferative effects on mesothelioma cell lines and efficiently abrogates their clonogenic growth. Exposure of sensitive cell lines with JQ1 results in decreased proliferative cell activity and induction of apoptosis. In addition to confirming the effect of JQ1, the BrdU also provided a guideline for treatment concentrations to be used in a further study.

CONCLUSION

This study has shown that BET domain proteins BRD2 and BRD4 are significantly altered in the tumors of patients suffering from MPM. Critically levels of BRD4 are significantly elevated in the tumors. JQ1 is a small molecule inhibitor of BRD4 and BRD2. This study has shown that JQ1 may be a potentially effective therapy for MPM *in vitro*.

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Research Article

Ad Hoc On-demand Distance Vector Inherent Techniques Comparison for Detecting and Eliminating the Black Hole Attack Nodes in Mobile *Ad Hoc* Network

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ABSTRACT

It is important to connect wirelessly a group of moving mobile nodes together in a static or dynamic form, to transfer digital data between them, this form known as a mobile *ad hoc* network. This private network can be used in different essential situations where it depends on each connected mobile node to deliver and pass the data between them, without any fixed access point or router. Unfortunately, there are different types of attacks that can affect these nodes, and steal or corrupt the data inside, one of these attacks called the black hole attack. In this paper, a compared study will be done between two major innovative techniques derived from the *ad hoc* on-demand distance vector routing protocol to avoid the black hole attack; the paper will compare the two techniques in delay, throughput and packet dropping efficacy.

Keywords: Black hole attack, extended data routing information technique, mobile *ad hoc* network, secure *ad hoc* on-demand distance vector routing protocol, trusted *ad hoc* on-demand distance vector

INTRODUCTION

Some scenarios or situation needs a group of moving nodes wirelessly connected together in a static or dynamic infrastructure form; this can be known as mobile *ad hoc* network (MANETs).[1] This form can broadcast or unicast data between each wireless mobile node separately or together. MANET can change its form dynamically depending on the moving nodes speed as shown in Figure 1; therefore, it shows better moving infrastructure to be used for different types of situations such as military, search and rescue, wireless sensor networks, and many more.[2] Where each node can move separately or with its private group to form the new infrastructure without the need of fixed access point or routers. Each node in MANET is responsible for cooperating with other nodes to send and receive the data smoothly using a different type of routing protocols (RPs) that are designed, especially for MANET.

There are three types of RPs for networking transmission operations and routing management, namely, proactive, reactive, and hybrid RPs.[3] Each protocol is depending on the size and the dynamic nature of the topology of MANET.[4] The proactive protocols basically are table driven, meaning. It depends on a specific table that used for routing decisions,[5] while reactive protocols are depending on an on-demand decision where it calculates the path needed for sending the data between the nodes on the same time that they need to transmit the data.[6] Hybrid protocols use both reactive and proactive techniques to deliver the data between the nodes.[7] Figure 2 shows the types of MANETs RPs.

It is essential to secure the data transmitted between the moving nodes to provide maximum security; unfortunately, wireless nodes have many security issues to be attacked.[8] One of these attacks called the black hole attack. In this paper, two major annotative techniques, namely, the trusted *ad hoc* on-demand distance vector (TAODV) RP technique and the extended data routing information (EDRI)-based technique will be compared and evaluated to prevent and detect the black hole attack.

This paper will be organized as follows: In section 1, an explanation will be shown for both black hole attack and it's two preventing techniques; section 3 will demonstrate a comprising between the two techniques; and finally, section 4 will conclude the paper.

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THE BLACK HOLE ATTACK AND AODV RP

In MANETs, all RPs have security issues, therefore, creating a robust RP to transfer data between the nodes without any security issues is difficult, due to many problems such as share link broadcast wireless channel, node's environment insecurity, the absence of center administration, the limitation of resources, and many more.[9] The essential requirements in any communication system to transfer data are integrity, confidentiality, and availability.[10] These requirements insurance the data will be intact without any eavesdroppers that can intercept or replace the transferred data. There are different types of attacks depending on the involvement of the attacker which can be classified into the passive attack and active attack. Moreover, each one of these attacks can be classified into external and internal attacks.[11]

The Black Hole Attack

One of the active attacks in MANET is the black hole attack, in this attack, one communicating node in the private network, after receiving the RREQ message from the sender node to form a path to the destination and start to transmit the data, replies with a RREP message with a highest sequence number compared to other nodes, forcing the sender to form a path through this node to the destination node, and transmits all the data through this path to that suspicious node, in the end when the suspicious node receives all the data, it will drop it all, without forwarding them to the neighbour nodes,[11] Figure 3 shows the fake RREP from the suspicious node. This attack can be considered a serious threat in any wireless private network because all the transmitted data are dropped without any succeed end to end delivery; therefore, it will increase the threshold of successful packet delivery to 100%.

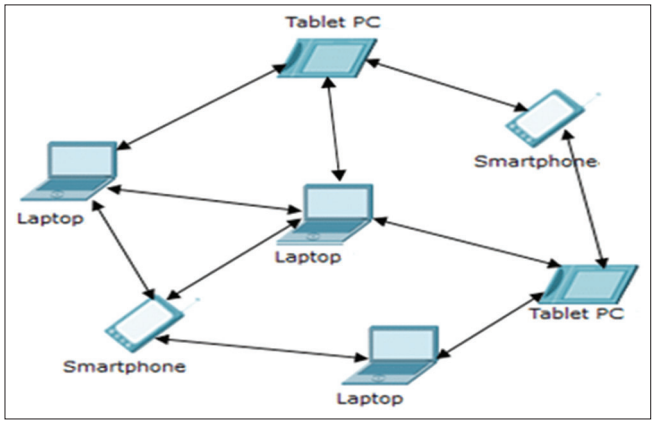

Figure 1: Mobile *ad hoc* network topology

AODV Inherent Black Hole Preventing Techniques

AODV is a reactive RP that starts a discovery route from source to destination when MANET topology changes and the source node want to transfer data, in AODV, each node in MANET stores and uses a routing table, to find the next neighbor node, which has the highest sequence number. In general, before the sender wants to transmit the data, it checks its routing table, therefore if there is a recent route to destination, it will send the data through that path, otherwise it will start a discovery route phase to find more recent route, where the sender will broadcast a request message RREQ to their neighbour nodes and then each node will reply with RREP message, and the highest sequence number node will participate in the path formed.[12]

Trusted AODV routing technique

This technique is built on the original AODV trusted protocol. When new MANET topology is established and a node wants to transmit information to a destination node, it will send a hello message to all other nodes to find the destination, each node after receiving the RREQ message will reply and respond to the sender node with their unique ID; after that, the sender node will be built a neighboring list consist of all nodes around its wireless range, which is stored in the node's memory. This table stores each node's ID in the range, also each node has a trust factor value stored in the table. When the value is small, it means that the node is suspicious, unreliable, and new to the network, and when the value is high, it means that the node is trusted and it was participating in old transmission path

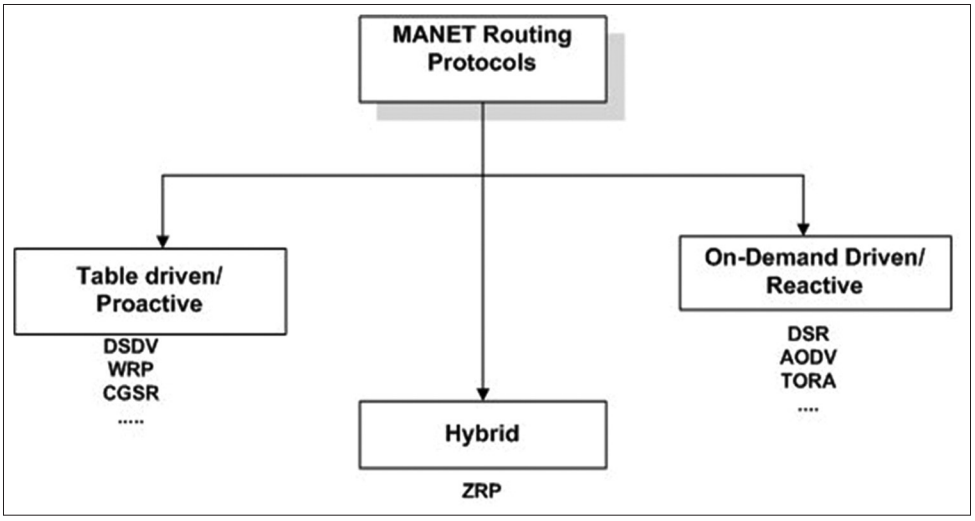

Figure 2: The types of mobile *ad hoc* networks routing protocols

before. Therefore, when the sender node broadcasts the RREQ message, the trusted value function will be used as a feature found in AODV RP to compute the level of trustiness for each node, and when each node receives the RREQ message, they will reply back directly to the sender, so it will be giving a trusting value. After that, when the node involved in a root establishment mechanism, the sender will verify this node by the forward route request RREQ to check its integrity and if the integrity is ok, the trusted value will be increased. After each node stores the trust value factor for intermediate nodes, it would become easier to detect the black hole nodes, so when the sender node will send the RREQ and establishes the path to the destination node, it will make sure that each intermediate node in the path has a higher trust factor, and other nodes with small trust factor will not participate in the future path for a definite time.[13] Figure 4 shows the trusting technique of AODV, where the nodes with higher trusting value are most reliable and with lower trusting value are unreliable.

EDRI technique

The second technique also is an extension from AODV RP; this approach uses a data routing information table (DRI) and an extended data routing information table (EDRI); it also uses a data control packet to detect and discover the suspicious nodes in MANET. Using the EDRI table, each node dynamically updates and stores their current and previous neighbors node's ID, the DRI table including two columns (from and through) and the black hole number (BHN), so when the middle nodes receive a packet from any neighbors' node, it writes 1 in the from column, but when the same node sends the packet to the neighbor note, it writes in the through column 1. When a neighbour node is detected as a black hole the flag of BHN number will be set to 1 and it will be set to 0 when there is no detection. Figure 5 shows the EDRI table.[14]

As known, any suspicious black hole node drops partially or fully all received packets; therefore, control data packets will be sent to check all the middle nodes in the path from source to destination. This packet contains node's id, next node hop NHN, and a random number that are constant for all data packets in that path, this control's data packet cannot be forwarded by a suspicious node. Another data control packet will be also used which can be transmittable by the suspicious node, to check which node did not respond to the previous control data packet. Figure 6 shows the composition of the data control packet.

After receiving all the RREP messages, the sender node will analyze and select the best-secured path to the destination, for transmitting the data packets and detect any suspicious black hole nodes in the path and bandit from future mapping.

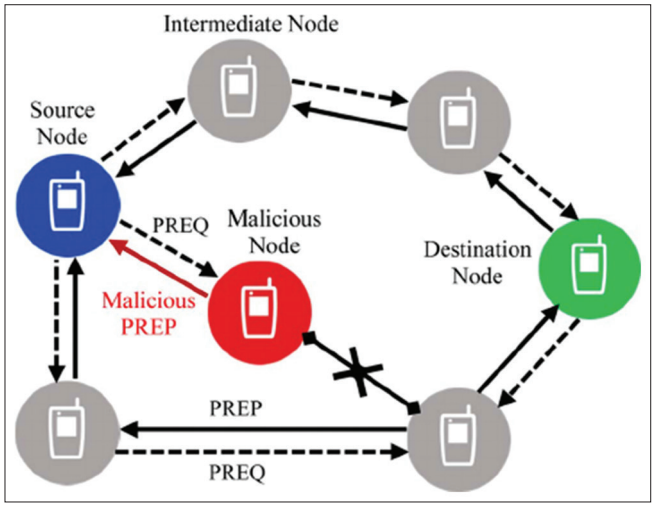

Figure 3: Mobile *ad hoc* network's black hole attack

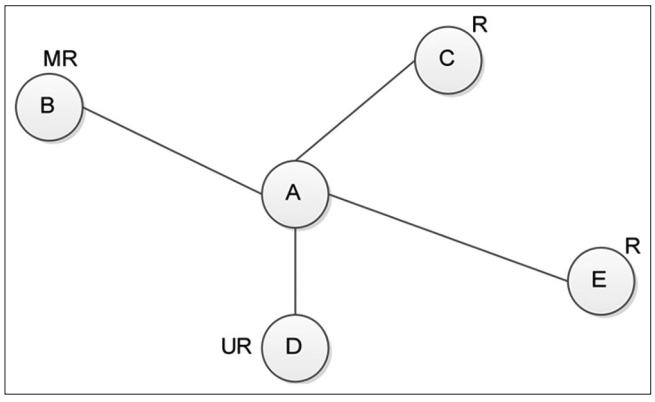

Figure 4: *Ad hoc* on-demand distance vector trusting technique

COMPARISON AND RESULTS

To evaluate the two techniques to prevent the black hole attack and isolate the suspicious nodes from MANET, a scenario was made to simulate the prevention of the attack; the same metric where used to evaluate the two algorithms, packet overhead, throughput, and delay was measured.

When the number of suspicious black hole nodes increases, the end-to-end delay will also be increased, due to the fact of packet dropping, renewal of path formation, and resending of the data message. All of these causes force the sender node to retransmit the packets until it gets acknowledgment from the receiving destination node.

In the trusted AODV (TAODV) technique, when the sender node requests a fresh route, a path to the destination node will be formed, and only the trusted node with higher trusting value will participate in the new path, the trusted nodes

Neighbor node's ID	Data routing information		BHN
	From	Through	
4	0/1	0/1	0/1
2	0/1	0/1	0/1

Figure 5: Example of extended data routing information table

Node_ID	NHN
Random_Number	

Figure 6: The component of the data control packet in extended data routing information technique

number in total are small; therefore, the end-to-end delay will increase. As a result, from the first running transmission, the number of trusted nodes cannot be increased, which causes the sender to use only the available trusted ones, so the delay will not be decreased rapidly overtime. Moreover, when the delay of the message increases, the packet dropping will also increase. Furthermore, the packet overhead will increase and the packet delivery ratio will be decreased due to the previous causes. Figure 7 shows how the number of trusted nodes values increase over multiple transmissions overtime.

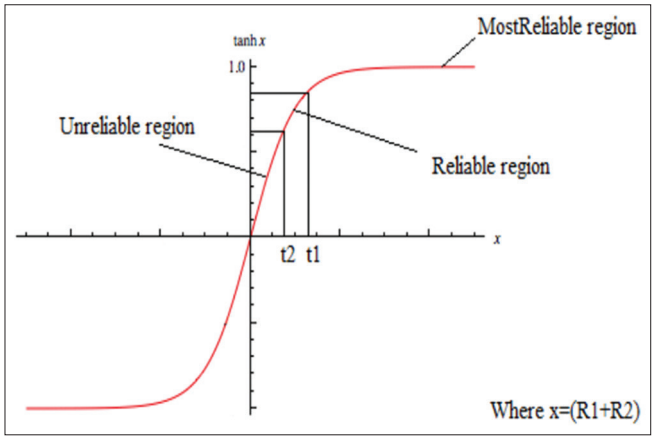

Figure 7: Graph represents the trusted nodes overtime

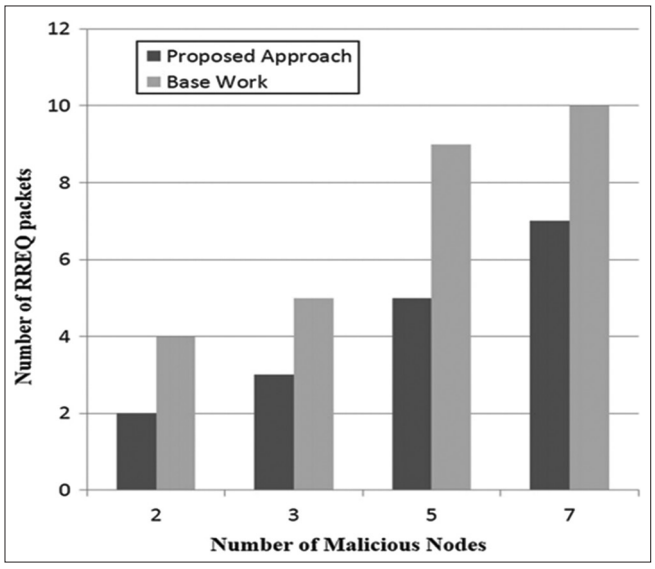

Figure 8: RREQ number evaluation

In EDRI technique, the sender node sends an RREQ packets to generate a safe path, by sending to the neighbor nodes the data control packet, the delay will increase at the beginning of transmission, after that, when all suspicious nodes detected, overtime the delay will be decreased rapidly, due to the fact that all the suspicious nodes were detected in the first running safe path, and no more RREQ packet needs to be broadcasted. Figure 8 shows the decreasing number of RREQ packets overtime.

Moreover, overtime when the number of trusted nodes increases due to EDRI table, the overall delay will be decreased. Moreover, the packet overhead will also be decreased overtime, due to the fact that the sender will reach more trustable nodes so connection packet will not be needed anymore between the neighbor nodes. Figure 9 shows the main comparison of the delay between the two techniques.

The throughput of the network was also compared, in the black hole attack, every single suspicious node sends an RREP to a source node and receives all the data and drops it from that sender, when the number of suspicious node increases, the number of dropping packet increases; therefore, the throughput will decrease. Moreover, when the number of suspicious nodes becomes equal or more than the sender nodes, the throughput will be equal to zero.

In the TAODV technique, the suspicious nodes will not participate in the safe path to the destination, due to the trust value factor, therefore, only the trusted ones will join the safe path, still, the trusted nodes number starting is lower than the normal ones, as a result less throughput and higher packet overhead.

In EDRI technique, all the suspicious nodes will be detected by each sender node in the first run; therefore, the number of

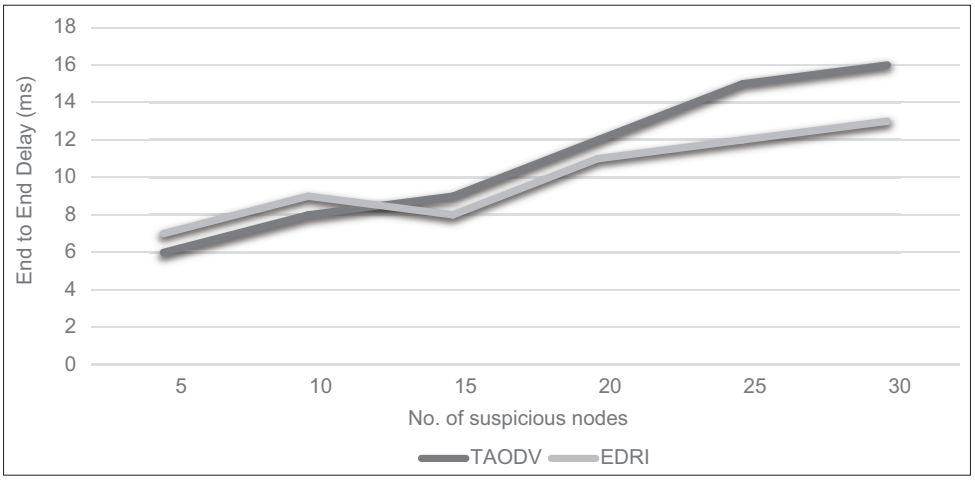

Figure 9: Delay comparison between trusted *ad hoc* on-demand distance vector and extended data routing information

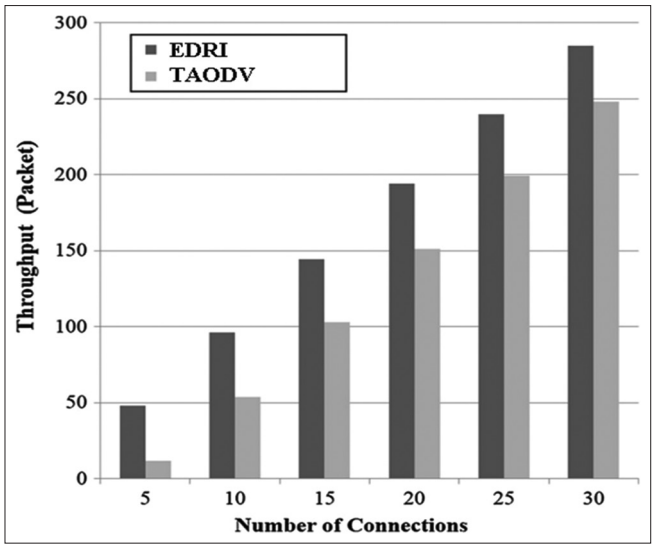

Figure 10: Throughput comparison between trusted *ad hoc* on-demand distance vector and extended data routing information

dropped packets will decrease, throughput will be higher in general, also by time, all the suspicious nodes will be detected, and they will be eliminated, therefore, the throughput will increase through time rapidly. Figure 10 shows the throughput for the two techniques.

CONCLUSION

In this paper, two annotative techniques were compared to detect and eliminate the black hole node attack in MANET, the paper compared three metrics; delay, packet overhead, and throughput.

As a conclusion, the TAODV technique shows a lower delay at the beginning of the transmission, due to the fact that no data control packet was used to detect the suspicious nodes, after that, the delay increases slowly, due to no elimination of suspicious nodes. While in the EDRI technique, the delay higher in the beginning then it decreases rapidly during the time.

In throughput, TAODV shows lower packet delivery compared to EDRI, while EDRI shows better throughput due to the elimination of suspicious nodes overtime, which increases the packet successful delivery.

Finally, both techniques show good delay and throughput to detect the problem of black hole attack nodes, but these techniques work only in one run transmission, new protocols needed to detect multiple runs from the 1st time.

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Cihan University-Erbil Scientific Journal (CUESJ)

Research Article

Detection and Evaluation of Effective of Digital Communication of Drug on Human Body

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ABSTRACT

Nowadays, there is a wide using of mobile and internet from various ages, due to some bad systems which they work to send signals (sound wave frequency) within the music or will be spread to affect the brain of the humankind. Therefore, this paper expresses the detection and evaluation of (the signal of sound frequency) which influence the human brain, it has been measured some frequency by programming in the mobile and found a trend to detect such frequencies and a way to produce a human being from it.

Keywords: Digital communication, digital drug, electromagnetic radiation

INTRODUCTION

The updated technology of communication (mobile and network) makes our social life more easy for communication and it will be active, but at the same time, it causes our lives many dangerous issues (such as electromagnetic radiation and sound pressure, especially ultrasonic waves) for all ages as well. Meanwhile, we need to point to specify the negative side of this new technique equipment and define all the waves with their bad usages. As we know the ear is a sound pressure receptor (or sensor) that influences our behavior (physically or physiology) because these techniques are getting inside houses without any our permission or observations. Whereas, the sense of hearing counts on the sensory receptors of the inner ear known as hair cells and the hair cells are extremely vulnerable and can be affected by disease, ageing and over-exposure to loud noise. Once destroyed, they do not regenerate.

Sound Properties and Characteristics[1]

It is the compression wave traveling down material bar. It is a physiological sensation of hearing and it looks like mechanical motion which is considered as longitudinal waves (number of consecutive rarefaction and consecutive compression as shown in Figure 1).

CHARACTERISTICS OF SOUND

Amplitude, Pitch, Frequency (n) =1/T, Period (T) =1/f, Wavelength (λ).

Speed (v), Quality, Tone, and A note.

Amplitude (A): It is a maximum displacement of particles vibration from their meaning poison and it is two types: (A) Louder sound (higher energy) and (B) soft sound. (It counts on wave distribution). Wavelength (λ): It is a distance between two consecutions compression and two consecutions rarefaction in the longitudinal frequency (n): It is a number of waves produced by a unit time and its unit is (hertz) n=1/T. Pitch: It is an interpretation of the frequency of a sound by the brain. It affects on the wave shape where the high pitch that means high frequency and amplitude and short wavelength (which gives a loud sound [or pitch]).

Period (T): It is a time duration of the wave (frequency) T=1/n or it is sound propagate on an intervals between two successive consecutions compression and two consecutions rarefaction.

Quality (Timbre) of the sound: It is a character helps us to make a different between two sound different sources but same pitch or loudness. Tone, it is a sound wave of single frequency. A note, it is a sound wave of the number of frequencies.

Speed (v): It is the ratio between distance (wavelength) and the time.

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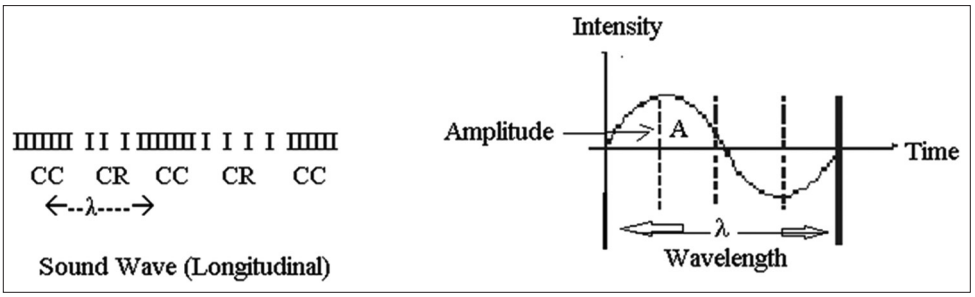

Figure 1: Characteristics of sound wave

Speed of sound v =Dis./Time = λ/T but n=1/T, then v=n λ

Wavelength: The distance between two consecutive compressions and two consecutive rarefactions in the long of the wave moving. The speed of the sound depends on type of the medium (material). There are several effectives of the sound (depending on sound pressure level as shown in Table 1) on humankind such as physic scaly, physiology, and behaviors.

Sound Effects

Digital communication affects humankind's body in many various methods such as drug detection, wireless communications, and various channels including several quantities of e-posts, email newsletters, social, targeted outreach to media, and health-care professionals in a bad way. These messages and communications announcement among people can be changed from new or appear risks related to concurrent conditions (for example, pregnancy or existing liver or kidney disease) or attentions about potential medication errors. Moreover, digital communication including issues influencing a great deal of patients, potentially serious or life-threatening injuries events, or medication errors that may result in serious or life-threatening conflicting answers. Whereas the digital communication influences directly on the behaviorism, mental and psychology of the person without feeling it because he/she will be detected automatically and this will affect humankind's mentality and it may lead to commit suicide as well.

The arriving narrative forms of communication increase information processing and increase the persuasiveness of messages and people become transported into a situation that can enhance emotions, attitudes, and behaviors.

As a result, it will be not easy to get rid of this detection which became part of his/her life everywhere and anywhere.

Digital Drugs[2]

More accurately called binaural beats which are sounds that can be capable of changing brain wave patterns and inducing an altered state of consciousness similar to that affected by taking drugs or achieving a deep state of meditation. Binaural beats occur when two tones with slightly different frequencies are played together. Without headphones, the slight difference in the two frequencies is perceived by the listener as a single tone that wavers slightly. Hence, headphones, however, the two tones are isolated and the listener hears each frequency clearly in a different ear. As the brain processes the two tones, it must take into account that the slight difference among the frequencies. As for the listener, this difference is perceived as rhythmic beats inside the head.

Table 1: Effective of sound level

Sound pressure level	Permissible exposure time
115 dB	0.468 min (≅30 s)
112 dB	0.937 min (≅1 min)
109 dB	1.875 min (<2 min)
106 dB	3.75 min (<4 min)
103 dB	7.8 min
100 dB	15 min
97 dB	30 min
94 dB	1 h
88 dB	4 h
82 dB	16 h

The brain processes rhythmic stimulus as electrical impulses. The goal of digital drugs is to purposely overcome the electrical impulses and encourage the listener's brain to synchronize its brain waves with the binaural beats. This synchronization which is achieved by selecting binaural tones within a particular frequency level is called frequency following response and is a part of a concept called entrainment. Entrainment, the synchronization of one biological rhythm to another is not a new concept. It forms the basis for many sorts of meditation and medical biofeedback.

Properties of Digital Drugs[3,4]

Frequencies are measured in units called hertz (Hz) by listening to two tones in difference falls within a particular Hz level, the listener hopes to achieve a particular mood or change in energy. For instance, if the listener wants to be very relaxed, he might choose to listen to a tone with 140 Hz in one ear and 145 Hz in the other. The listener's brain would perceive the viciousness between the two frequencies (5 Hz) and adjust the listener's brain waves accordingly. If the listener wants to be energized, however, he might choose to listen to 130 Hz in one ear and 150 Hz in the other. A difference of 20 Hz in frequencies would produce a various state of mind as shown in Table 2.

Sound Spectrum Measuring[5]

Using a special sound meter program (within mobile application) which has been measured by some frequency spectrum sound for ultrasonic to supersonic (where the range of humane hear from 20 Hz to 20,000 Hz), some frequencies are out of the hearing range, as shown in Figure 2, starting from 2A for frequency before 20 Hz to 2 D after 20,000 Hz.

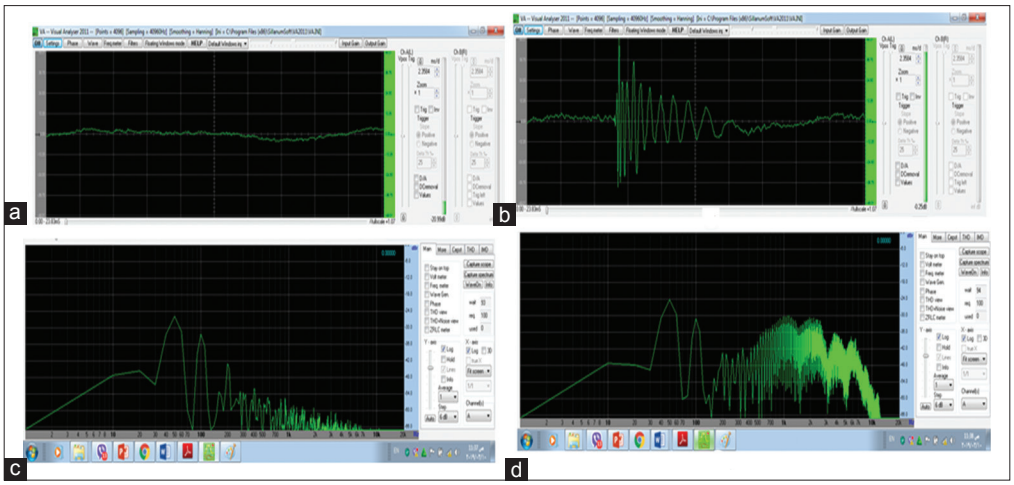

Figure 2: (a-d) Sound frequencies spectrum

Table 2: State of sound frequency

Type	Frequency range (hZ)	State of mind
Delta	0.5 Hz – 4	Deep sleep
Theta	4 Hz – 8	Drowsiness (also first stage of sleep)
Alpha	8 Hz – 14	Relaxed but alert
Beta	14 Hz – 30	Highly alert and focused

Table 3: Noises can cause damage to wear hearing

Type	Noise value (dB)	Period
A	85 dB	Regular and prolonged exposures to noise at or above 85 dB (averaged 8 h/day)
B	100 dB	Regular and prolonged unprotected exposures of more than 15 min/day risk permanent hearing loss
C	110 dB	Regular and prolonged unprotected exposures of more than 1.5 min/day risk permanent hearing loss

That means there are a lot of frequencies affect on human's body without hearing it.

RESULTS AND DISCUSSION[6,7]

1. Using mobile has a side effect which is the microwave radiation as well as the effect of ultrasound waves
2. The effective of microwaves is physically, but the effective of ultrasound is physiological, physical, and behavior
3. The output of microwave effective will appear after a long period while the effective of ultrasound will appear during a short period
4. Digital well-being considerations. When optimizing digital health, multiple health-relatedcomponents can be taken into consideration.[6]
5. The effective is usually different from person to other because it counts on the followingfactors:
   a. The time period that the person stay under the frequency of sound wave as shown in Table 3
   b. The behavior and situation of the person during the effective
   c. The power and pressure level of the sound. Table 1 shows the level and time of affecting (exposure time)
   d. Using headphone or without (because using headphone is effected more).

CONCLUSION

Digital well-being is a term used by health professionals, researchers, and device manufacturers to describe the concept of humans interact with technology. The experience should support mental and/or physical health in a measurable way. The goal of improving digital well-being is to design technology in such a way that it promotes healthy use and proactively assists the user to maintain a healthy lifestyle.[4,6]

Using a digital communication (mobile, laptop, and iPad) with internet, the user certainly will receive and affect the unwanted sound frequency wave; of course, the user effective counted on the factors that mentioned above.

We should observe the kids and children to be sure; they are using the digital instruments on right ways by:

a. They should use free beneficial programs of any digital drug
b. Control and check the children not to join or purchasing materials or playing games witha bad group
c. Observe the children's behavior in any serious forward motion (such as dancing, doing something, or chatting) and you should know in which programming he is busy with it.

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Cihan University-Erbil Scientific Journal (CUESJ)

Research Article

Effect of *Isatis* spp. Extraction on the Growth of *Aspergillus niger* and *Candida albicans*

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ABSTRACT

In this study, the crude extraction of *Isatis* spp. (*Isatis tinctoria, Isatis busichaina,* and *Isatis lusitanica*) was investigated for their antifungal activity. Each of methanol, ethanol, and H_2O was used as a solvent. Due to find the most effective part of each species, the plant parts were used separately. The aim of this research was to determine the natural products effect on two common pathogenic fungi *Aspergillus niger* and *Candida albicans.* The results show that most of used plants have significant effect on both used fungal species. Extracted each of flower, *I. tinctoria* was showed the best results comparing to the other used species. Extracted leaf and flower of *I. tinctoria* by methanol were showed the best result on *A. niger* and *C. albicans.* The growth zone was around 90 mm for control and 61 mm at 75% concentration of methanolic and ethanolic extraction. The flower was for followed by the stem. Depend on the results, the methanol was showed highest number, then ethanol and the lowest inhibition zone area was for H_2O extraction.

Keywords: Antifungal activity, *Aspergillus niger* and *Candida albicans, Isatis* spp., pathogenic fungi

INTRODUCTION

A latest study presented that tertiary care clinics are consuming antimicrobial and antifungal agents improperly. This rising trend is an ever-increasing threat to the health of humans who are currently at risk of dying from previously fixable infections. Unluckily, drug resistance (adaptation of the drug target site, drug modification, or restricted drug penetration) improves at a pace quicker than new drug development.[1] This sensation is also happening in the pathogenic microorganism *Candida albicans*, an otherwise commensal kinds found in and on the human body.[2] Modern antifungal agents are less active against *C. albicans* now than when they were first introduced because *Candida* species have developed resistance to these agents. In addition, research by the major pharmaceutical companies in the development of antifungal agents has been on the decline due to low coming back on investment.[3]

MATERIALS AND METHODS

Plant Extracts

Three methods were used to extract water, ethanolic, and methanolic, according to several studies, with some modification as needed and as follows:[4]

Aqueous Extraction

Hot water extraction was performed by mixing 40 g of plant form with 160 ml of distilled water twice (i.e., 1:4 weight:volume), heated to 80–90°C, then stirred and left to cool down and mixed together then placed in refrigerator for 24 h, which was then administered through several layers of medical gauze, and again administered by Buchner funnel using filter papers Whatman No.1 to dispose of the non-powdered parts and fibers to obtain the raw liquid extract and then put it in the rotary evaporator device at a temperature not exceeding 40°C, working on the basis of evaporation under pressure and then put the extracted in the shaker incubator at a temperature of 30–35°C. After the drying of the extract,

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it was kept in a sealed containers in the freezer (−4°C) and wrote the information on each until use.

Ethanolic Extraction

Prepare the ethanol extract by mixing 20 g of plant powder in 200 ml of absolute ethanol with stirring (i.e., 1:10 weight:volume) and then leave the mixture in the refrigerator for 24 h. Then, the extract was filtered through several layers of gauze and then filtered for 2nd time using Whatman No.1 filter papers to remove the non-powdered plant parts and the remaining fibers, then put in the rotary evaporator device at 40°C and after evaporation of the alcohol obtained a thick layer of the extract and then placed in the shaker incubator shaker incubator at 25–30°C. After drying the extract, it was kept in a sealed container in a freezer with writing the information on each until use.

Methanolic Extraction

Prepare the methanol extract by mixing 20 g of plant powder in 200 ml of absolute methanol with stirring (i.e., 1:10 weight:volume) and then leave the mixture in the refrigerator for 24 h to soak. Then, the extract was filtered through several layers of gauze and then filtered for 2nd time using Whatman No.1 filter papers to remove the non-powdered plant parts and the remaining fibers, then put in the rotary evaporator device at 40°C and after evaporation of the methanol obtained a thick layer of the extract and then placed in the shaker incubator shaker incubator at 25–30°C. After drying the extract, it was kept in a sealed container in a freezer with writing the information on each until use.

Media Preparation

The above studied media were prepared according to the information on the package by the manufacturer company[5] then sterilized at 121°C and 15 lb/kg^2 for 15 min. The medium was distributed in Petri dishes (diameter 9 cm), and the dishes were left under the temperature of the laboratory until the hardening of the medium in sterilizer hood. A quantity of distilled water was also sterilized for use as a substitute for extracts in control transactions (ddH_2O).

Fungi

Two types of fungus obtained from the media center in Erbil-Iraq for diagnosing the disease with mention the strain type were used in this study.

- *Aspergillus niger* ATCC 16404
- *C. albicans* ATCC 10231

Preparation of Petri dishes to Test Extracts against Fungus

For this study, concentration method was used by mixing with the medium. The fungal isolates were planted in Petri dishes on the potato dextrose agar media, mixed with three different concentrations of plant extracts that mentioned in previously, then incubated the dishes in the incubator at 25 ± 2°C and 37 ± 2°C. For example, *Candida* species need a temperature of between 25 ± 2°C and 37 ± 2°C for optimal growth according to the nature of their growth to fungus or semi-yeast. The process of incubation continued for 10 days. The readings were taken every 3 days according to replicates, influenced by concentrations of plant extracts.[6,7]

Statistical Analysis

GLM-univariate analysis was applied to the arithmetic means obtained in SPSS with 95% confidence interval. If the difference was significant ($P \leq 0.05$), the homogeneity groups were determined by Tukey B test. The analytical test was done separately for each solvent type (hot water, ethanol, and methanol) to show the effect of the samples part (flower, leaf, and stem) and extract concentration (25%, 50%, and 75%).

RESULTS AND DISCUSSION

The antifungal compound is one of the vital substances in pharmaceutical sciences that can find in the natural medicinal plants. This needs to identify the activity of the samples compound by different parameters. In this study, the antifungal activities of extracts obtained with solvents that vary from non-polar to polar were investigated. The results were took after the growth was rich the optimum point of two types of used fungi in optimum incubation condition. The growth of the control Petri dishes was 90 mm for both. The following table is showed the effect of *Isatis tinctoria* on *A. niger* ATCC 16404 and *C. albicans* ATCC 10231 growth. The best results were with flower extraction by methanol with 75% concentration against *A. niger* ATCC 16404, following by the stem with methanolic extraction 75% against *A. niger* ATCC 16404 too. In this table, all of the methanolic extraction had significant effect on both fungi *A. niger* ATCC 16404 and *C. albicans* ATCC 10231 [Table 1]. The second table is showed the effect of *Isatis busichaina* on *A. niger* ATCC 16404 and *C. albicans* ATCC 10231 growth. The best results were with leaf extraction by methanol with 75% concentration against *C. albicans* ATCC 10231, following by the flower with methanolic extraction 75% against *A. niger* ATCC 16404. In this table, all of the methanolic extraction had significant effect on both fungi *A. niger* ATCC 16404 and *C. albicans* ATCC 10231 [Table 2]. However, Table 3 shows the effect of *Isatis lusitanica* on *A. niger* ATCC 16404 and *C. albicans* ATCC 10231 growth. The best results were with flower extraction by methanol with 75% concentration against *A. niger* ATCC 16404, following by the flower with methanolic extraction 75% against *C. albicans ATCC 10231*. In this table, all of the methanolic extractions had significant effect on both fungi *A. niger* ATCC 16404 and *C. albicans* ATCC 10231 [Table 3].

Depend on the results, the significant results were obtained from the extraction of methanol and ethanol for *I. tinctoria* in all concentrations, plant parts, and both types of used fungi. The aqueous extraction has less effect in correlation with ethanol and methanol [Table 4]. The inhibition effects of the extracts of *I. tinctoria* obtained from leaf, stem, and flower parts using different solvents on the two different strains of pathogenic fungi. The results were compared with the each to find the correlation. The aqueous leaf extracts were showed significant results on *A. niger* ATCC 16404, also the flower was showed the significant results by ethanol extraction for *C. albicans* ATCC 10231, although the flower extracts by methanol were showed the significant results for *A. niger* ATCC 16404. Other differences are shown in Table 4.

Table 1: Fungi test results of *I. tinctoria*. (Control=90 mm)

Plant	Plant part	Strains	Growth of fungi (mm)								
			Water concentration			Ethanol concentration			Methanol concentration		
			25%	50%	75%	25%	50%	75%	25%	50%	75%
I. tinctoria	Flower	*C. albicans*	85.75 (0.96)	82.75 (0.50)	80.25 (0.96)	81.00 (0.82)	79.00 (0.82)	72.50 (1.29)	73.00 (1.15)	71.75 (0.50)	62.00 (1.41)
		A. niger	84.00 (0.82)	82.50 (1.29)	79.00 (0.82)	81.25 (0.96)	80.00 (0.82)	78.00 (1.41)	69.75 (0.50)	68.00 (0.82)	61.25 (0.50)
	Leaf	*C. albicans*	84.50 (2.38)	80.75 (0.96)	79.50 (1.29)	79.75 (1.26)	78.75 (0.96)	77.75 (0.96)	70.5 (0.58)	69.25 (0.50)	62.00 (1.41)
		A. niger	84.00 (0.82)	81.00 (0.82)	79.25 (0.96)	80.25 (1.71)	78.75 (0.96)	77.50 (0.58)	70.25 (0.50)	68.50 (1.00)	62.75 (0.50)
	Stem	*C. albicans*	84.75 (0.96)	82.25 (2.06)	80.50 (0.58)	82.00 (0.82)	80.50 (0.58)	73.00 (1.83)	73.50 (1.29)	70.25 (0.50)	63.25 (1.50)
		A. niger	84.50 (1.29)	82.75 (0.96)	80.00 (0.82)	81.25 (0.50)	79.25 (0.96)	78.50 (0.58)	70.75 (0.96)	69.50 (1.29)	61.75 (0.50)

I. tinctoria: Isatis tinctoria, C. albicans: Candida albicans, A. niger: Aspergillus niger

Table 2: Fungi test results of *I. busichaina*. (Control=90 mm)

Plant	Plant part	Strains	Growth of fungi (mm)								
			Water concentration			Ethanol concentration			Methanol concentration		
			25%	50%	75%	25%	50%	75%	25%	50%	75%
I. busichaina	Flower	*C. albicans*	84.75 (0.96)	80.25 (1.26)	79.50 (1.29)	80.00 (0.82)	78.50 (0.58)	76.00 (1.41)	71.00 (0.82)	70.00 (1.63)	63.00 (1.83)
		A. niger	83.50 (1.29)	79.75 (0.96)	77.75 (2.22)	80.00 (0.82)	78.25 (0.50)	76.00 (0.82)	70.75 (0.96)	68.00 (0.82)	61.5 (1.29)
	Leaf	*C. albicans*	83.25 (0.96)	79.50 (1.29)	77.00 (1.83)	80.00 (0.82)	79.00 (0.82)	75.50 (1.29)	71.00 (1.15)	66.75 (2.06)	61.00 (0.82)
		A. niger	83.25 (0.96)	80.50 (0.58)	77.25 (1.71)	80.75 (0.96)	78.50 (0.58)	74.25 (0.50)	70.75 (0.50)	66.25 (0.50)	63.00 (1.83)
	Stem	*C. albicans*	84.00 (0.82)	80.75 (1.50)	77.25 (1.50)	81.75 (0.96)	80.00 (0.82)	78.25 (0.96)	72.5 (2.38)	70.50 (0.58)	68.50 (0.58)
		A. niger	84.75 (1.50)	82.25 (1.26)	79.25 (0.96)	81.75 (0.96)	80.50 (0.58)	79.25 (0.96)	71.75 (0.96)	70.00 (0.82)	69.00 (1.41)

I. busichaina: Isatis busichaina, C. albicans: Candida albicans, A. niger: Aspergillus niger

Table 3: Fungi test results of *I. lusitanica*. (Control=90 mm)

Plant	Plant part	Strains	Growth of fungi (mm)								
			Water concentration			Ethanol concentration			Methanol concentration		
			25%	50%	75%	25%	50%	75%	25%	50%	75%
I. lusitanica	Flower	*C. albicans*	83.25 (0.96)	81.50 (1.29)	79.00 (0.82)	80.50 (0.58)	79.50 (1.29)	78.00 (0.82)	70.00 (0.82)	67.75 (0.50)	64.50 (1.29)
		A. niger	83.25 (0.96)	80.75 (0.96)	79.00 (0.82)	80.50 (0.58)	78.25 (1.71)	76.75 (0.96)	68.50 (1.29)	66.50 (1.00)	63.75 (1.71)
	Leaf	*C. albicans*	82.25 (1.71)	81.75 (1.71)	79.50 (1.29)	80.00 (0.82)	79.00 (0.82)	76.75 (0.96)	69.75 (0.96)	67.50 (0.58)	66.50 (1.00)
		A. niger	82.00 (0.82)	80.25 (0.96)	79.50 (0.58)	80.25 (0.50)	78.25 (1.71)	77.50 (1.29)	68.25 (0.96)	65.75 (0.96)	65.25 (0.50)
	Stem	*C. albicans*	84.50 (0.58)	82.25 (0.50)	80.50 (1.29)	81.50 (1.29)	80.75 (0.96)	79.00 (0.82)	72.00 (0.82)	70.50 (0.58)	69.00 (0.82)
		A. niger	83.50 (1.29)	80.75 (0.96)	80.25 (1.26)	81.50 (0.58)	80.00 (0.82)	79.00 (0.82)	72.00 (0.82)	70.25 (0.96)	69.50 (0.58)

I. lusitanica: Isatis lusitanica, C. albicans: Candida albicans, A. niger: Aspergillus niger

The inhibition effects of extracts of *I. busichaina* obtained from leaf, stem, and flower parts using different solvents on the two different strains of pathogenic fungi. The results were compared with the each to find the correlation. For *C. albicans*

ATCC 10231, each of aqueous leaf extraction, ethanol extraction of flower and leaf, and leaf extraction by methanol was showed the significant result correlations. For *A. niger* ATCC 16404, the aqueous extraction of flower and leaf, the ethanol extraction of flower and leaf, and methanol extraction of flower and leaf too was showed the significant results. The total result statistics shown in Table 5.

The inhibition effects of the extracts of *I. lusitanica* obtained from leaf, stem, and flower parts using different solvents on the two different strains of pathogenic fungi. The results were compared with the each to find the correlation. For *C. albicans* ATCC 10231, each of flower and leaf of aqueous, ethanol, and methanol extraction was showed the significant results. For *A. niger* ATCC 16404, the ethanol and methanol extraction of flower and leaf was showed the significant results. The total result statistics is shown in Table 6. Some factors have to be measured to select a solvent for extracting herbal medicine. About 65–75% methanol extraction at room temperature for 15 h is practical for routine analytical study. Although, it is not fully active to extract other plants with same procedure because of dissimilarities in the biological compositions. This method is appropriate for controlling a large number of fixed samples and needs no special equipment.[8]

The present study proved that methanol and ethanol showed best results than water extracts. Our study was in accordance with several studies.[9] Tryptanthrin, indole-3-acetonitrile, and p-coumaric acid methylester were identified by physical and spectral data (MS, NMR) and by comparison with authentic synthesized samples of these compounds. Tryptanthrin has already been isolated from *I. tinctoria* and characterized as an antidermatophytic.[10] Phenylpropanoids mainly comprised flavonoid and lignan metabolites, have been considered as the primary antiviral constituents, which

Table 4: Fungi arithmetic values and Tukey B statistical analysis results of *I. tinctoria*

	***C. albicans* concentration**				***A. niger* concentration**				**Tukey B**
	Plant part	**25%**	**50%**	**75%**	**Tukey B**	**25%**	**50%**	**75%**	
Water	Flower	85.75 (0.96)	82.75 (0.50)	80.25 (0.96)	A	84.00 (0.82)	82.50 (1.29)	79.00 (0.82)	AB
	Leaf	84.50 (2.38)	80.75 (0.96)	79.50 (1.29)	A	84.00 (0.82)	81.00 (0.82)	79.25 (0.96)	B
	Stem	84.75 (0.96)	82.25 (2.06)	80.50 (0.58)	A	84.50 (1.29)	82.75 (0.96)	80.00 (0.82)	A
	Tukey B	A	B	C		A	B	C	
Ethanol	Flower	81.00 (0.82)	79.00 (0.82)	72.50 (1.29)	B	81.25 (0.96)	80.00 (0.82)	78.00 (1.41)	A
	Leaf	79.75 (1.26)	78.75 (0.96)	77.75 (0.96)	A	80.25 (1.71)	78.75 (0.96)	77.50 (0.58)	A
	Stem	82.00 (0.82)	80.50 (0.58)	73.00 (1.83)	AB	81.25 (0.50)	79.25 (0.96)	78.50 (0.58)	A
	Tukey B	A	B	C		A	B	C	
Methanol	Flower	73.00 (1.15)	71.75 (0.50)	62.00 (1.41)	B	69.75 (0.50)	68.00 (0.82)	61.25 (0.50)	B
	Leaf	70.50 (0.58)	69.25 (0.50)	62.00 (1.41)	C	70.25 (0.50)	68.50 (1.00)	62.75 (0.50)	A
	Stem	73.50 (1.29)	70.25 (0.50)	63.25 (1.50)	A	70.75 (0.96)	69.50 (1.29)	61.75 (0.50)	A
	Tukey B	A	B	C		A	B	C	

I. tinctoria: Isatis tinctoria, C. albicans: Candida albicans, A. niger: Aspergillus niger

Table 5: Fungi arithmetic values and Tukey B statistical analysis results of *I. busichaina*

	Plant part	***C. albicans* concentration**			**Tukey B**	***A. niger* concentration**			**Tukey B**
		25%	**50%**	**75%**		**25%**	**50%**	**75%**	
Water	Flower	84.75 (0.96)	80.25 (1.26)	79.50 (1.29)	A	83.50 (1.29)	79.75 (0.96)	77.75 (2.22)	B
	Leaf	83.25 (0.96)	79.50 (1.29)	77.00 (1.83)	B	83.25 (0.96)	80.50 (0.58)	77.25 (1.71)	B
	Stem	84.00 (0.82)	80.75 (1.50)	77.25 (1.50)	AB	84.75 (1.50)	82.25 (1.26)	79.25 (0.96)	A
	Tukey B	A	B	C		A	B	C	
Ethanol	Flower	80.00 (0.82)	78.50 (0.58)	76.00 (1.41)	B	80.00 (0.82)	78.25 (0.50)	76.00 (0.82)	B
	Leaf	80.00 (0.82)	79.00 (0.82)	75.50 (1.29)	B	80.75 (0.96)	78.50 (0.58)	74.25 (0.50)	B
	Stem	81.75 (0.96)	80.00 (0.82)	78.25 (0.96)	A	81.75 (0.96)	80.50 (0.58)	79.25 (0.96)	A
	Tukey B	A	B	C		A	B	C	
Methanol	Flower	71.00 (0.82)	70.00 (1.63)	63.00 (1.83)	B	70.75 (0.96)	68.00 (0.82)	61.5 (1.29)	B
	Leaf	71.00 (1.15)	66.75 (2.06)	61.00 (0.82)	C	70.75 (0.50)	66.25 (0.50)	63.00 (1.83)	B
	Stem	72.5 (2.38)	70.50 (0.58)	68.50 (0.58)	A	71.75 (0.96)	70.00 (0.82)	69.00 (1.41)	A
	Tukey B	A	B	C		A	B	C	

I. busichaina: Isatis busichaina, C. albicans: Candida albicans, A. niger: Aspergillus niger

Table 6: Fungi arithmetic values and Tukey B statistical analysis results of *I. lusitanica*

	Plant part	*C. albicans* concentration			Tukey B	*A. niger* concentration			Tukey B
		25%	50%	75%		25%	50%	75%	
Water	Flower	83.25 (0.96)	81.50 (1.29)	79.00 (0.82)	B	83.25 (0.96)	80.75 (0.96)	79.00 (0.82)	A
	Leaf	82.25 (1.71)	81.75 (1.71)	79.50 (1.29)	B	82.00 (0.82)	80.25 (0.96)	79.50 (0.58)	A
	Stem	84.50 (0.58)	82.25 (0.50)	80.50 (1.29)	A	83.50 (1.29)	80.75 (0.96)	80.25 (1.26)	A
	Tukey B	A	B	C		A	B	C	
Ethanol	Flower	80.50 (0.58)	79.50 (1.29)	78.00 (0.82)	B	80.50 (0.58)	78.25 (1.71)	76.75 (0.96)	B
	Leaf	80.00 (0.82)	79.00 (0.82)	76.75 (0.96)	B	80.25 (0.50)	78.25 (1.71)	77.50 (1.29)	B
	Stem	81.50 (1.29)	80.75 (0.96)	79.00 (0.82)	A	81.50 (0.58)	80.00 (0.82)	79.00 (0.82)	A
	Tukey B	A	B	C		A	B	C	
Methanol	Flower	70.00 (0.82)	67.75 (0.50)	64.50 (1.29)	B	68.50 (1.29)	66.50 (1.00)	63.75 (1.71)	B
	Leaf	69.75 (0.96)	67.50 (0.58)	66.50 (1.00)	B	68.25 (0.96)	65.75 (0.96)	65.25 (0.50)	B
	Stem	72.00 (0.82)	70.50 (0.58)	69.00 (0.82)	A	72.00 (0.82)	70.25 (0.96)	69.50 (0.58)	A
	Tukey B	A	B	C		A	B	C	

I. lusitanica: Isatis lusitanica, C. albicans: Candida albicans, A. niger: Aspergillus niger

probably contribute to the outstanding pharmacological activity of Radix isatidis.[11,12]

It is distinguished that a solvent system for extraction is selected according to the purpose of extraction such as preparation or analysis, the nature of interested components, the physicochemical properties of the matrix, the availability of reagents and equipment, cost, and safety concerns.[13] The solvent systems included complete ethanol at 25°C by different methods, 95% ethanol, ethanol–water for 10 min,[14] methanol–water, methanol–HCl (1.0 N, 85:15, v/v), and H_2O.

In another study that done by in 2012,[15] using the ethanol under pressure, extraction condition may be a well choice for viable productions, meanwhile, ethanol may be reused and fewer solvent left over is generated through production. However, extraction with ethanol may be a blameless means to make effective extracts for analysis and research from a minor number of samples.

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