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FORWARD

Dear Colleagues,

IJST was a fruitful effort issued by the International Centre for Advancement of Sciences and Technology – ICAST, which tries to take part in both globalization and revolution in information and communication technologies, because S&T development becoming not only the key elements of economic growth and industrial competitiveness, but also essential for improving the social development, the quality of life and global environment. ICAST took then a decision to establish a scientific alliance with TSTC (Tharwa for scientific Training & Consultations) and this alliance comes to support the efforts towards publishing IJST.

Today, we announce a new issue of our journal, that is the second issue from the thirteen volume of IJST, June , 2018.

Finally, I hope that all significant figures of sciences whom joined the editorial board, the researchers, and the readers of our journal will keep IJST between their eyes and contribute in continuing its journey, with their remarks, valuable recommendations and their researching outcomes.

Thanks a lot for all who support IJST.

Editor-in-Chief

IJST

Abdul Jabbar Al- Shammari

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Determination of microbial limits for pharmaceutical dosage forms by chromogenic agars

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ABSTRACT

Pharmaceutical products have been routinely contaminated with several types of microorganisms right during and after manufacture. Microbial contamination refers to the presence of undesirable microbes or their metabolites in products. The microbial limit was assigned to determine the allowed number of microorganisms in pharmaceutical products. The aim of the current study was to use known chromogenic agar to determine objectionable microorganisms instead of conventional methods already used. Four chromogenic agars namely; Candida chromogenic agar, Salmonella chromogenic agar, Pseudomonas chromogenic agar, and Urinary tract infections chromogenic agar were used. Thirty-six pharmaceutical samples, represented various dosage forms were tested by a conventional technique and chromogenic agar methods. Out of 36 samples tested by chromogenic agar 11, 7, 9 and 18 samples have given more than 100 CFU/ml and were positive on Candida chromogenic agar, Salmonella chromogenic agar, Pseudomonas chromogenic agar, and on urinary tract infection chromogenic agar respectively. Comparison with the conventional method, less number of bacteria were obtained. The study concluded that the use of chromogenic agars in the determination of microbial limit was time and cost reducing in addition to accuracy.

Keywords: chromogenic agar methods, conventional technique, Microbial contamination

INTRODUCTION

Different types of pharmaceutical products have been routinely contaminated with fungi and bacteria species right during and after manufacture (1-4). While most of the microorganisms contaminating nonsterile pharmaceutical products, may be nonpathogenic but they pose problems as agents of spoilage (5-9). Many of these organisms may also become opportunistic pathogens in compromised individuals.

The importance of and potential for microbial contamination of pharmaceuticals is widely recognized in the pharmaceutical industry and attempts to safeguard products from contamination in the manufacturing process include, among others, good manufacturing practices and accurate methods to detect the contaminants. The microbial limit technique was used by most pharmacopeias to determine the allowed number may be presences in pharmaceutical forms (United States Pharmacopeia (USP-61,62). Most regulatory agencies and drug manufacturers have attempted to set suitable microbial limit standards for incoming raw materials and excipients used in drug product manufacturing. In limiting the microbial load of these products, one can reduce the risk for product spoilage, product recalls, and potential patient infection relative to the product's end use.

Chromogenic media have been used for diagnosis of UTI and uropathogens (10-11), normal intestinal flora (12), and for detection of Enterobacteriaceae (13-15) and candida pathogens (16,17).

By this study, a trial to introduce the chromogenic agars technique was done as fast and accurate tool for determination of bioburden in pharmaceutical dosage forms. Chromogenic agars have been used for rapid diagnosis in clinical microbiology (18). As a result, microbial limit testing of pharmaceutical dosage forms was less time consuming, since counting of colonies formed and identification of microorganisms was performed at the same time. Other advantages of using chromogenic agar media in microbial limit testing include; Chromogenic agar media is a highly specialized growth medium for microbiologists to aid in the isolation and identification of certain types of pathogenic microorganisms.

MATERIALS AND METHODS

Pharmaceutical dosage form samples:

A total of thirty- six pharmaceutical dosage forms was found to be contaminated by various species of bacteria and fungi selected from our previous studies (7,8,19). The original samples were collected from various pharmacies at Amman, Jordan. The tested Samples representative for syrup (6), tablets (6), eye drops (10), eye ointment (10), Agar (Laboratories Conda, Cat; 1493 .1, Spain), Salmonella Chromogenic Agar (Laboratories Conda, Cat; 1122 .1, Spain) and Urinary Tract Infections Chromogenic Agar [UTIC] (Laboratories

baby cream (2) and hair gel (2). All pharmaceutical dosage forms samples were throughout their expiration dating period.

Chromogenic agars:

Four chromogenic agars were used namely, Candida Chromogenic Agar (CCA)(Laboratories Conda, Cat; 1382 .1, Spain) for detection of *C.albicans*, *C.tropicalis*, *C.krusei* and other Candida species, Pseudomonas Chromogenic Agar (PCA)(Laboratories Conda, Cat; 1493 .1, Spain) for detection of Pseudomonas, Salmonella Chromogenic agar (SCA) (Laboratories Conda, Cat; 1122 .1, Spain) for detection of Salmonella including *S.typhi* and Urinary Tract Infections Chromogenic Agar (UTIC) (Laboratories Conda, Cat; 1424 .1, Spain) for detection of *Escherichia coli*, *Proteus mirabilis*, *Enterobacter aerogenes*, *Staphylococcus aureus*, *Enterococcus faecalis* and *Klebsiella pneumoniae*.

Preparation of samples:

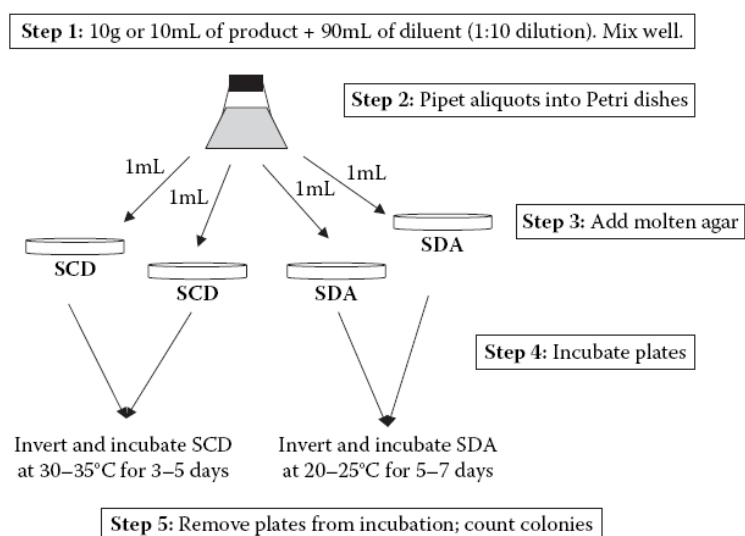
The samples were prepared as described in USP 61 (United States Pharmacopeia (USP-61). Briefly; 10 g of each tablet sample was milled using sterile mortar and pestle and then dissolved in sterile distilled water up to 100 ml. Ten ml of syrup or eye drop samples were mixed (separately) with sterile distilled water up to 100 ml, and 10 g of ointments, baby cream, and hair gel samples (separately) with addition of 5 ml of tween 80 and using water bath on 37 °C were dissolved in sterile distilled water up to 100 ml. All dispersions were left to settle for five minutes to dislodge possible microbial cells and to separate the solid particles and supernatants to be used in further tests. Sterile distilled water was used as a negative control.

Determination of microbial limit:

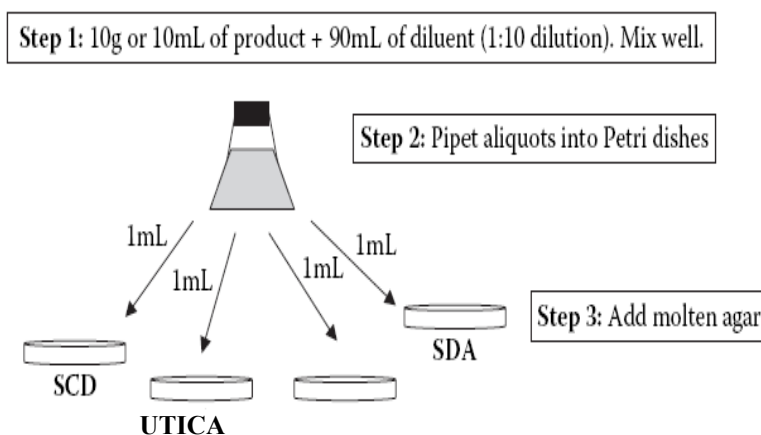
conventional method: A small portion of samples (0.2 ml) were streaked on the surface of nutrient media, soya bean casein digest and Sabouraud dextrose agar in duplicate (Clontz, 2010). Samples streaked on nutrient agar and soya bean casein digest plates were incubated at 35 °C for five days. While samples streaked on Sabouraud dextrose agar plates were incubated at 25 °C for seven days. After incubation, the plates which showed growth were subcultured to isolate colonies for testing. Colonies were first tested using Gram stain for bacteria and lactophenol blue for fungi (figure 1-a).

Chromogenic agars method: A small portion of samples (0.2 ml) were streak on the surface of Candida Chromogenic Agar (Laboratories Conda, Cat; 1382 .1, Spain), Pseudomonas Chromogenic

Conda, Cat; 1424 .1, Spain) (figure 1-b). Plates were incubated at 37°C and observed to determine colony coloration and morphology at 24, 48, and 72 hrs. (16).



(a): The USP microbial limit test



(b): The proposed method of microbial limit test using chromogenic agar media

Figure (1-a): Original microbial limit test according to the USP. Figure (1-b): microbial limit test using chromogenic agar media. (A modified scheme of microbial limit test according to USP chapter 61).

Presumptive identification and enumeration:

A-Conventional method: Phenotypic identification of bacteria and fungi isolates were further tested by Gram stain, lacto- phenol cotton blue (LPCB) and biochemical tests reaction. Remal RapiD system (Thermo Fisher Scientific, USA) was used to identify *Staphylococcus* species, *Enterobacteriaceae* and *Candida* species (20,21).

appeared as magenta, while the colonies of *Pseudomonas aeruginosa* appeared in magenta color and all other micro-organisms were inhibited. The colors color of *Staphylococcus aureus*, white

B-Chromogenic agar: Determination of the colonies colors on four chromogenic media was performed according to manufacturer's instructions. The identification is based on chromogenic substrates incorporated into the media to detect certain bacterial and *Candida* enzymes. Colors of *Candida krusei* colonies appeared as purple pink, while colonies of *Candida parapsilosis* and *Candida glabrata* showed light white – purple colonies. Colors of *Salmonella* species colonies

cream; *Klebsiella pneumonia*, dark blue; *Proteus mirabilis*, light brown; *Enterococcus faecalis*, light blue; and *Escherichia coli*, pink.

Statistical analysis:

One-way ANOVA statistical analysis method was used to test differences in microbial limits by conventional and chromogenic agars methods (22).

RESULTS

Thirty-six pharmaceutical samples were tested for microbiological contamination by chromogenic agar

media; Candida chromogenic agar (CA), Salmonella chromogenic agar (SCA), Pseudomonas chromogenic agar (PCA), and Urinary tract infections chromogenic agar (UTICA).

The number of colonies were enumerated by chromogenic agar media and was multiplied by ten (dilution factor). Tables (1-6) illustrate results obtained by chromogenic agar test of the pharmaceutical dosage form samples and by conventional method taken from previous studies.

Table (1): Microbial enumeration of tablets dosage form counted by chromogenic agars and conventional methods

Dosage form/code	Chromogenic agars				Conventional
	CCA	SCA	PsCA	UTICA	
Ri3Fa	<100 CFU/g	<100 CFU/g	1.1×10^2 CFU/g	> 100 CFU /g	5×10^2 CFU/g
Ri3Fb	1.1×10^2 CFU/g	14×10^2 CFU/g	<100 CFU/g	> 100 CFU /g	5×10^2 CFU/g
Ri2a	< 100 CFU /g	<100 CFU/g	2×10^2 CFU/g	<100 CFU/g	5×10^2 CFU/g
Ri2b	< 100 CFU /g	<100 CFU/g	<100 CFU/g	<100 CFU/g	<100 CFU/ml
Ri1a	1.1×10^2 CFU/g	17×10^2 CFU/g	2×10^2 CFU/g	<100 CFU/g	5×10^2 CFU/g
Ri1b	2.6×10^2 CFU/g	12×10^2 CFU/g	2.9×10^2 CFU/g	<100 CFU/g	5×10^2 CFU/g

Table (2): Microbial enumeration of eye drops dosage form counted by chromogenic agars and conventional methods

Dosage form/code	Chromogenic agars				Conventional
	CCA	SCA	PsCA	UTICA	
A1a	<100 CFU/ml	<100 CFU/ml	<100 CFU/ml	> 100 CFU /ml	<100 CFU/ml
A1b	< 100 CFU /ml	<100 CFU/ml	<100 CFU/ml	<100 CFU/ml	<100 CFU/ml
A2a	< 100 CFU /ml	<100 CFU/ml	<100 CFU/ml	> 100 CFU /ml	<100 CFU/ml
A2b	< 100 CFU /ml	<100 CFU/ml	<100 CFU/ml	> 100 CFU /ml	<100 CFU/ml
P1a	2.4×10^2 CFU/ml	6.6×10^2 CFU/ml	<100 CFU/ml	> 100 CFU /ml	5×10^2 CFU/ml
P1b	< 100 CFU /ml	<100 CFU/ml	2.4×10^2 CFU/ml	> 100 CFU /ml	5×10^2 CFU/ml
P2a	< 100 CFU /ml	<100 CFU/ml	<100 CFU/ml	> 100 CFU /ml	2.4×10^2 CFU/ml
P2b	< 100 CFU /ml	<100 CFU/ml	<100 CFU/ml	> 100 CFU /ml	2.4×10^2 CFU/ml
A3Fa	< 100 CFU /ml	<100 CFU/ml	<100 CFU/ml	<100 CFU/ml	2.4×10^2 CFU/ml
A3Fb	< 100 CFU /ml	<100 CFU/ml	1.6×10^2 CFU/ml	> 100 CFU /ml	7×10^2 CFU/ml

Table (3): Microbial enumeration of syrups dosage form counted by chromogenic agars and conventional methods

Dosage form/code	Chromogenic agars				Conventional
	CCA	SCA	PsCA	UTICA	
L1a	1.5×10^2 CFU/ml	1.8×10^2 CFU/ml	2.4×10^2 CFU/ml	> 100 CFU /ml	<100 CFU/ml
L1b	1.5×10^2 CFU/ml	2×10^2 CFU/ml	2.4×10^2 CFU/ml	> 100 CFU /ml	5×10^2 CFU/ml
L2a	< 100 CFU /ml	<100 CFU/ml	<100 CFU/ml	<100 CFU/ml	<100 CFU/ml
L2b	< 100 CFU /ml	<100 CFU/ml	<100 CFU/ml	<100 CFU/ml	<100 CFU/ml
L3Fa	1.8×10^2 CFU/ml	<100 CFU/ml	<100 CFU/ml	> 100 CFU /ml	5×10^2 CFU/ml
L3Fb	< 100 CFU /ml	<100 CFU/ml	<100 CFU/ml	<100 CFU/ml	<100 CFU/ml

Table (4): Microbial enumeration of eye ointment dosage form counted by chromogenic agars and conventional methods

Dosage form/code	Chromogenic agars				Conventional
	CCA	SCA	PsCA	UTICA	
O3a	1.8×10^2 CFU/ml	<100 CFU/ml	<100 CFU/ml	> 100 CFU /ml	<100 CFU/ml
O3f	< 100 CFU /ml	<100 CFU/ml	<100 CFU/ml	<100 CFU/ml	<100 CFU/ml
P3Fa	< 100 CFU /ml	<100 CFU/ml	<100 CFU/ml	> 100 CFU /ml	<100 CFU/ml
P3Fb	< 100 CFU /ml	<100 CFU/ml	<100 CFU/ml	> 100 CFU /ml	<100 CFU/ml
O2a	< 100 CFU /ml	<100 CFU/ml	<100 CFU/ml	> 100 CFU /ml	5×10^2 CFU/ml
O2b	< 100 CFU /ml	<100 CFU/ml	<100 CFU/ml	> 100 CFU /ml	5×10^2 CFU/ml
O4a	< 100 CFU /ml	<100 CFU/ml	<100 CFU/ml	> 100 CFU /ml	5×10^2 CFU/ml
O4b	< 100 CFU /ml	<100 CFU/ml	<100 CFU/ml	> 100 CFU /ml	2.4×10^2 CFU/ml
O1CAE	< 100 CFU /ml	<100 CFU/ml	<100 CFU/ml	> 100 CFU /ml	2.4×10^2 CFU/ml
O2CAE	< 100 CFU /ml	<100 CFU/ml	<100 CFU/ml	> 100 CFU /ml	7×10^2 CFU/ml

Table (5): Microbial enumeration of hair gels dosage form counted by chromogenic agars and conventional methods

Dosage form/code	Chromogenic agars				Conventional
	CCA	SCA	PsCA	UTICA	
V2a	<100 CFU/ml	<100 CFU/ml	<100 CFU/ml	> 100 CFU /ml	<100 CFU/ml
V2b	<100 CFU/ml	<100 CFU/ml	<100 CFU/ml	> 100 CFU /ml	> 100 CFU /ml

Table (6): Microbial enumeration of baby cream dosage form counted by chromogenic agars and conventional methods

Dosage form/code	Chromogenic agars				Conventional
	CCA	SCA	PsCA	UTICA	
V1a	<100 CFU/ml	<100 CFU/ml	<100 CFU/ml	> 100 CFU /ml	<100 CFU/ml
V1b	<100 CFU/ml	<100 CFU/ml	<100 CFU/ml	> 100 CFU /ml	<100 CFU/ml

Chromogenic agars showed an accurate number of microbial limits. Statistical analysis reveals that there were no differences in total number, but chromogenic agar shows more details for each group of microorganisms. The bioburden test of samples did not give any surprising results since the samples were selected as contaminated dosage forms.

Since these samples were selected as bioburden samples and have unacceptable limits of microorganisms, identification of microorganism's presences in each pharmaceutical dosage form showed the superiority of chromogenic agars over conventional methods. Bacterial species which were not identified in the conventional method, they identified clearly on chromogenic agar (figures 2-4).

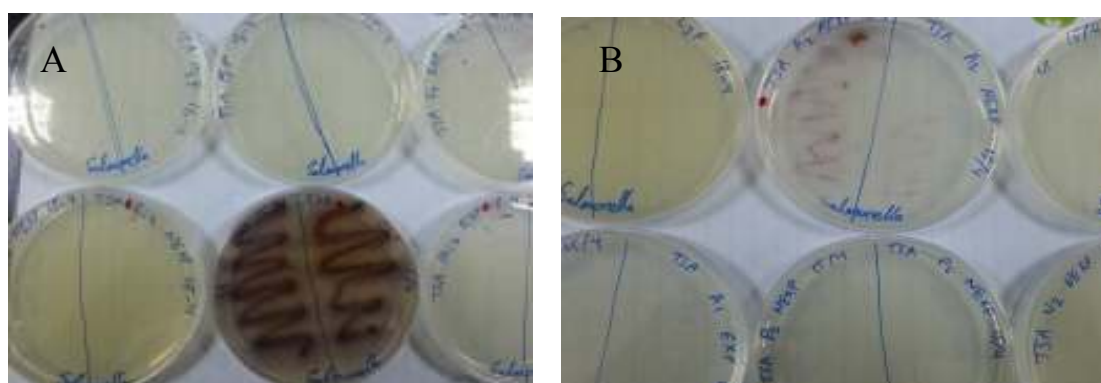


Figure (2): Salmonella chromogenic agar (SCA), A: the magenta color of samples P1a, P1b and Ri2. B: for samples A2a and A2b

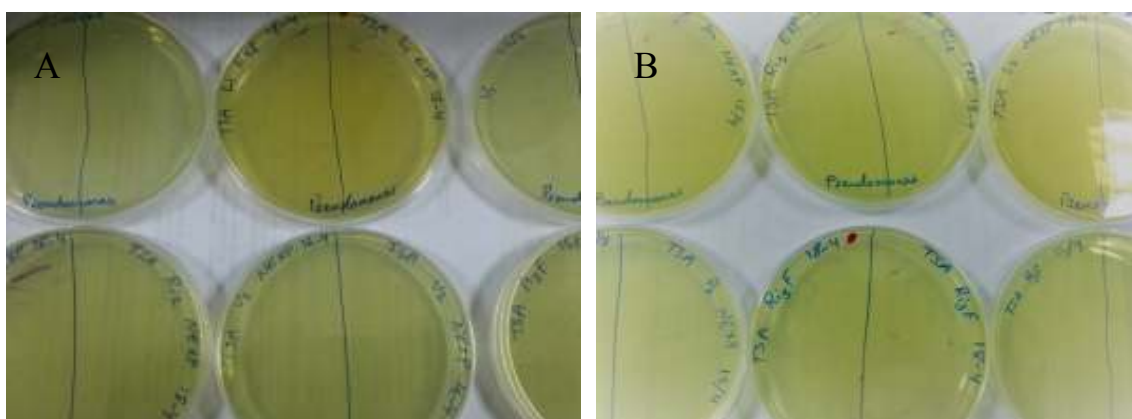


Figure (3): Pseudomonas chromogenic agar (PCA). A: the magenta color of samples L1a, L1b and Ri2a. B: for samples Ri1 and Ri3F

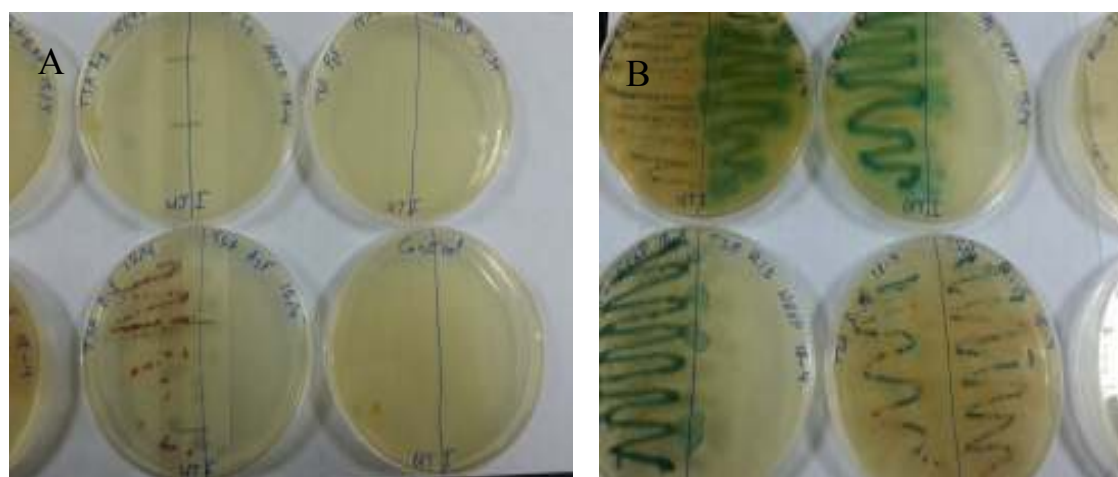


Figure (4): UTI chromogenic agar (UTICA). A; *E. coli* (A3F) pink in color. B top; *Staphylococcus aureus* (Ri1a & b) (creamy) and *Klebsiella pneumoniae* (dark blue) (A1a); B bottom; *Enterococcus faecalis* (Ri3)(light blue)

In table (7) which showed the presumptive identification of microorganisms contaminated tablets, in addition to *Candida* species identified by conventional method, bacterial species such as *Salmonella typhi*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* in samples Ri3Fa, Ri3Fb, Ri1a and Ri1b (table 7), eye drops samples A1a, P1a, P1b and P2b (table 8), and eye ointments pharmaceutical dosage form O3a, O2a, O2b, O4b and O2CAE (table 9). On other compresses identification were noticed in Ri2a (table 7), A2a, A2b and A3Fa and Fb (table 8). Two syrups samples were identified as *Candida krusei* (L1a and L1b) instead of *Candida parapsilosis*, *Escherichia coli* instead of *Salmonella* species and *Pseudomonas*

aeruginosa instead of *Candida parapsilosis* and *Candida glabrata* (table 10). Colors of *Candida krusei* colonies appeared as purple pink, while colonies of *Candida parapsilosis* and *Candida glabrata* showed light white – purple colonies. The same compresses were noticed in table 8 (samples

P3Fa, P3Fb, and O1CAE). No microbes were identified in hair gels (table 11) and baby creams (not seen in tables).

Table (7): Presumptive identification of tablet dosage form by chromogenic agars

Dosage form code	Identification by chromogenic gar	Identification by conventional
Ri3Fa	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> , <i>Salmonella typhi</i> , <i>Pseudomonas aeruginosa</i> , <i>Staphylococcus aureus</i>	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> ,
Ri3Fb	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> , <i>Salmonella typhi</i> , <i>Pseudomonas aeruginosa</i> , <i>Staphylococcus aureus</i>	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> , <i>Staphylococcus aureus</i>
Ri2a	<i>Pseudomonas aeruginosa</i>	<i>Staphylococcus aureus</i>
Ri2b	<i>Salmonella typhi</i>	<i>Salmonella species</i>
Ri1a	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> , <i>Salmonella typhi</i> , <i>Pseudomonas aeruginosa</i> , <i>Staphylococcus aureus</i> , <i>Klebsiella pneumoniae</i> ,	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> , <i>Staphylococcus aureus</i>
Ri1b	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> , <i>Salmonella typhi</i> , <i>Pseudomonas aeruginosa</i> , <i>Staphylococcus aureus</i> , <i>Klebsiella pneumoniae</i> ,	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> , <i>Staphylococcus aureus</i>

Table (8): Presumptive identification of eye drops dosage form by chromogenic agars

Dosage form code	Identification by chromogenic agar	Identification by conventional
A1a	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> , <i>Staphylococcus aureus</i> , <i>Klasiella pneumoniae</i> ,	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> ,
A1b	<i>Candida parapsilosis</i> , <i>Candida glabrata</i>	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> ,
A2a	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> , <i>Pseudomonas aeruginosa</i> , <i>Salmonella typhi</i> , <i>Proteus mirabilis</i>	<i>Staphylococcus aureus</i>
A2b	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> , <i>Salmonella typhi</i> , <i>Pseudomonas aeruginosa</i> , <i>Proteus mirabilis</i>	<i>Salmonella species</i>
P1a	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> <i>Salmonella typhi</i> , <i>Pseudomonas aeruginosa</i> , <i>Staphylococcus aureus</i> ,	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> , <i>Staphylococcus aureus</i>
P1b	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> <i>Salmonella typhi</i> , <i>Pseudomonas aeruginosa</i> , <i>Staphylococcus aureus</i> ,	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> , <i>Staphylococcus aureus</i>
P2a	<i>Enterococcus faecalis</i>	<i>Enterococcus faecalis</i>
P2b	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> , <i>Staphylococcus aureus</i>	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> ,
A3Fa	<i>Enterococcus faecalis</i>	<i>Escherichia coli</i>
A3Fb	<i>Pseudomonas aeruginosa</i> , <i>Escherichia coli</i>	<i>Escherichia coli</i>

Table (9): Presumptive identification of eye ointment dosage form by chromogenic agars

Dosage form code	Identification by chromogenic agar	Identification by conventional
O3a	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> , <i>Staphylococcus aureus</i> , <i>Klasiella pneumoniae</i> ,	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> ,
O3f	<i>Candida parapsilosis</i> , <i>Candida glabrata</i>	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> ,
P3Fa	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> , <i>Pseudomonas aeruginosa</i> , <i>Salmonella typhi</i> , <i>Proteus mirabilis</i>	<i>Staphylococcus aureus</i>
P3Fb	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> , <i>Salmonella typhi</i> , <i>Pseudomonas aeruginosa</i> , <i>Proteus mirabilis</i>	<i>Salmonella species</i>
O2a	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> <i>Salmonella typhi</i> , <i>Pseudomonas aeruginosa</i> , <i>Staphylococcus aureus</i> ,	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> , <i>Staphylococcus aureus</i>
O2b	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> <i>Salmonella typhi</i> , <i>Pseudomonas aeruginosa</i> , <i>Staphylococcus aureus</i> ,	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> , <i>Staphylococcus aureus</i>
O4a	<i>Enterococcus faecalis</i>	<i>Enterococcus faecalis</i>
O4b	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> , <i>Staphylococcus aureus</i>	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> ,
O1CAE	<i>Enterococcus faecalis</i>	<i>Escherichia coli</i>
O2CAE	<i>Pseudomonas aeruginosa</i> , <i>Escherichia coli</i>	<i>Escherichia coli</i>

Table (10): Presumptive identification of syrups dosage form by chromogenic agars

Dosage form code	Identification by chromogenic agar	Identification by conventional
L1a	<i>Candida krusei</i> , <i>Salmonella typhi</i> , <i>Pseudomonas aeruginosa</i> , <i>Staphylococcus aureus</i>	<i>Candida parapsilosis</i> ,
L1b	<i>Candida krusei</i> , <i>Pseudomonas aeruginosa</i> , <i>Staphylococcus aureus</i>	<i>Candida parapsilosis</i> , <i>Staphylococcus aureus</i>
L2a	<i>Staphylococcus aureus</i>	<i>Staphylococcus aureus</i>
L2b	<i>Escherichia coli</i>	<i>Salmonella species</i>
L3Fa	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> <i>Pseudomonas aeruginosa</i> , <i>Staphylococcus aureus</i> ,	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> , <i>Staphylococcus aureus</i>
L3Fb	<i>Pseudomonas aeruginosa</i>	<i>Candida parapsilosis</i> , <i>Candida glabrata</i> ,

Table (11): Presumptive identification of hair gels dosage form by chromogenic agars

Dosage form code	Identification by chromogenic agar	Identification by conventional
V2a		<i>Candida parapsilosis</i> , <i>Candida glabrata</i> ,
V2b		<i>Candida parapsilosis</i> , <i>Candida glabrata</i> ,

Colors of *Salmonella* species and *Pseudomonas aeruginosa* appeared as magenta on SCA (figure 2) and PCA respectively, while the color of bacterial species grown on UTICA shows variable color; *Staphylococcus aureus*, white cream; *Klebsiella pneumoniae*, dark blue; *Proteus mirabilis*, light brown; *Enterococcus faecalis*, light blue; and *Escherichia coli*, pink (figure 3).

DISCUSSION AND CONCLUSION

In this study, a total of 36 nonsterile pharmaceutical dosage forms delivered to the user by retail pharmacies at Amman city, Jordan were examined for objectionable microorganisms and subjected to identification by chromogenic agars instead of conventional method described in USP chapter 61 and 62. In general, chromogenic agar technique provides both, enumeration and identification, at the same time since it has proven more sensitive and specific than other conventional media. Besides, chromogenic media provide results within 24-48 hrs of incubation. Thus, it is also considered a time-saving technique than conventional method which may take 5 to 7 days. authors mentioned that chromogenic agars were superior then conventional methods in detection of *Salmonella* species (14), MRSA (23,24), urinary tract infection pathogens (10), and differentiation of Vancomycin-Resistant *Enterococcus faecium* and *Enterococcus faecalis* isolates (25,26). Chromogenic agar was used in parallel with a molecular technique like PCR and found no significant differences when PCR was compared with culture on chromogenic media after broth enrichment (23,27). All these findings encourage us to used chromogenic agars for enumeration and detection of non-sterile pharmaceutical dosage form because most pharmacopeias do not require that non-sterile pharmaceutical preparation to be sterile, but most pass tests for absences of objectionable microorganisms such as *Escherichia coli*, *Salmonella spp.*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Candida albicans*.

The important question is that why we try to add an additional technique for identification of microorganisms contaminated non-sterile preparation? because the harmonized microbial limits tests only address the “absence of specified microorganisms” and leave the determination of the absence of objectionable microorganisms in capable hands of each company’s (Scott, 2005), while the presences of microorganisms in pharmaceutical preparation have a physical and chemical deterioration of medication and needs to identified each microorganism contaminated pharmaceutical

preparation’s, especially in developing countries (6-8, 28-30).

According to USP chapter 61, the microbiological testing includes an identification of colonies found during the total aerobic plate count test, but the important to identifying all isolates from either both total plate count testing and enrichment testing, and that the chromogenic agars are capable to identify numerous microorganisms depend upon the chromogenic substrate. Obviously, the non-sterile pharmaceutical preparations may be acceptable to identify isolates when testing show high levels as shown in tables (1-5) in the current study.

Thus, this study concluded that the use of chromogenic agar in microbial limit tests is considered very useful in enumeration and detection of microorganisms better than the conventional methods.

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Epidemiological and Parasitological Studies on Hydatidosis in Kirkuk Province, Iraq

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ABSTRACT

The current study was conducted to investigate the prevalence of Hydatidosis in Kirkuk city, during the period from August 2016 to September 2017. The study involved the accident host (human), intermediate host (sheep, goats and cows) and definite host (dogs), as reported by human health office and veterinary service department. Results revealed that the female had the highest percentage (57.4%), comparison with male (42.6%). Most infected females were housewife's (45%) aged 31-60. The most affected organ was liver which have multiple cysts. Out of 600 sheep, 100 goats and 200 cows slaughter in Kirkuk slaughter house, the echinococcosis infection were 14%, 9% and 7 % respectively. A separate finding of 50 stray dogs shows 14% infections. The economic loses of humans and animals reached about 160 million USD.

Keywords: Hydatidosis, slaughter house, echinococcosis infection.

INTRODUCTION

Echinococcosis is an endemic disease in Iraq, since Iraq regards as agricultural country and there are huge number of stray dogs lives in the farms or attended the sharped and framer. Echinococcosis is a zoonotic disease transmitted to human being through contaminated food with dogs faces (1). The old name of disease was liver filled with water, as mention in books written by Aristo and Epicrot (2). Most developing countries were suffered from this disease and spent a lot of money for treatment and control of disease (3). Previous studies in Iraq indicated that the disease caused huge losses among human and animals, 1000 causes annually in human each year, and about 4.5-45%, 5.4-40%, 2.2-35% and 33.6-50% among sheep, goats, cow and camels respectively (4-13). Dogs are the definite host of disease, in Iraq, most of these dogs are stray dogs, 25-85% of these dogs were infested by echinococcosis (14). Although several studies conducted in Iraq, but the epidemiological picture still unclear, because most studies were concentrated in four governorates, namely Baghdad, Mosul, Basra and Erbil (4, 9-13, 15-17). Iraq needs a broad strategy include all provinces, in order to control of the disease. The current study aimed to investigated the prevalence of disease in Kirkuk city and surrounding area, since several points reveals that the hydatidosis present in Kirkuk observed in human internal organ during surgical operations, and occurrence of cysts in animals observed in slaughter houses.

MATERIALS AND METHODS

Human cases:

Human hydatid cysts were estimated through the medical records in surgery departments/ Kirkuk hospitals. The reports included the age, gender, occupation, address and affected organs.

Animals cases:

Field visits to slaughter houses and unauthorized places deals with the animals slaughtering in Kirkuk city, reveals that about 600 heads of sheep, 100 heads of goats and 200 heads of cattle's slaughter daily in the city. The presences of hydatid cysts in internal organs were reported such as: livers, kidneys, lungs and intestine. The number of hydatids cyst in each organ were counted depend upon the presences of protoscoleces in cyst fluid (12,18). The nature of metacestodes that consist of the germinal layer surrounded by the laminated layer were reported.

Dogs' hydatids:

The small intestine of fifty stray dogs were removed as soon as possible after death and tied at both ends as described by Deplazes and Eckert (19), and examined quickly, as the parasite can be digested within 24 hrs. The contents were empty in large plastic dish containing water and the inner layer of intestine were scraped for worm investigation. Number of worms were counting in each dog small intestine according to methods described by Romig *et.al*, (18).

Economic losses estimation:

The economic losses were estimated according to international standard (20), which includes: Diagnostic tests costs, Cost of surgical operation, Cost of resident of patient on the hospital and Cost of recovery period after operation before resume back to work. According to survey study which distributed to physician in hospitals, the estimated cost was determined. In order to obtained accurate cost, the authors distributed questioners directly to physicians and to hospitals. Financial losses were estimated by multiply the total number of infected animal's times percentage of affected livers and lungs. The results were multiplied for the price of sheep, goats and cow's livers.

Human economic estimation:

The questioners asked physician about the cost of diagnosis and treatment of hydatids'. The cost also included the residency in hospitals and number of days spent out of work for workers.

RESULTS

Human infections:

Table (1) reveals that number of females infected superior than males in Kirkuk providence. The percentage reached 57.4% and 42.6% in females and males respectively, Statistical analysis significant with regards to gender. Age of patients with hydatid cysts were distributed according to age shows that susceptibility to infection between 60-70 years. The highest percentage was in females between 30-60 years old, while the highest percentage in males were between 40-60. Statistical analysis according to Chi square shows significate differences ($P < 0.005$). According to occupational of patients, table (2) shows that mainly the housewives were the highest (45%) followed by students, farmer, workers, shoppers, teacher's, officers and children -infected in various percentage. Table (3) reveals the positions of cysts in body organs of patients. Eighty-three cysts in the livers of patients (96%) and 1% in lung, while 2 cysts in other organs in three patients (3%).

Table (1): Distribution of hydatidosis among gender and age of human patients

Age/year	Male	%of infected	Female	% of Infected	Total number	% of infected
0-10	2	3.2	3	3.5	5	5.7
11-20	3	3.5	4	4.6	7	8.0
21-30	4	4.6	5	5.7	9	10.4
31-40	6	6.9	9	10.3	15	17.3
41-50	8	9.2	14	16.1	22	25.3
51-60	10	11.5	12	13.7	22	25.3
61-70	4	4.6	3	3.5	7	8.0
Total	37	42.6	50	57.4	87	100

Table (2): Distribution of human hydatidosis according to their occupations

Occupation	Number of infected	% of infected
Housewife's	39	45
Students	15	17
Farmer	11	13
Laborers	7	8
Shopkeepers	6	7
Teacher	2	2
Officer	4	5
Children	3	3
Total	87	100

Table (3): Percentage of infected organs with hydatidosis in Kirkuk city

Infected organs	Number of infected	% of infected
Liver (right lobe)	65	75
Liver (left lobe)	9	10.3
Liver (right 7 left lobes)	9	10.3
Lungs and liver	3	3.4
lungs	1	1
Total	87	100

Financial losses due to hydatids:

The financial and economic losses due to hydatidosis involved two types of losses; predicate and unperidicate losses, an example for number two type represented by reduce productivity of individual's before, during and after operation. Predicated losses have been estimated according to criteria mention above and reach 1000 USD per governmental officer and 1400 USD per non-governmental officer. According these costs the total cost of infection by hydatidosis for 87 individuals in Kirkuk city equal 8700.00 USD, the amount of money not included the unpredicted losses.

Animal losses due to hydatidosis:

Post-mortem examination of 600 sheep, 100 goats, 200 cows slaughter at Kirkuk slaughter-house reveals that 86 head of sheep (14%), 9 heads of goats (9%) and 14 head of cattle's (7%) were infected (table 4). Sheep, goats and cattle's infected livers representative 45.2%, 51.3% and 30.4% respectively, while infected lungs representative 30.4%, 26.6%, 31.5% respectively. Combination liver and lungs infected were seen in 24.4% of sheep, 22.1% of goats and 26.1% of cattle's. Statistical analysis reveals significance at level of ($P < 0.001$) in distribution of hydatid cysts in different organs, infected livers were the highest percentage in number of hydatid cysts. The percentage of mature cyst found in visceral organs of sheep, goats and cattle were 71.6%, 52.5% and 44.8% respectively, the rest of cysts were either immature or calcificated.

Table (4): Distribution of hydatid cysts in slaughter sheep, goats and cattle

	Sheep	Goats	Cattle
Number of examined	600	100	200
Number of infected	86	9	14
% of infection	14	9	7
Number of examined cysts	1376	113	188
% of fertile cyst	71.6	52.5	44.8
% of infertile cyst	11.3	28.1	35
% of calisification cysts	17.1	19.4	20.2
% infected liver	45.2	51.3	42.4
% infected lungs	30.4	26.6	31.5
% infected both	24.4	22.1	26.1

Estimation of financial losses in animals due to hydatidosis:

The estimation depends upon the damaged livers and lungs, because livers were destroyed in both cases, if the price of health sheep or cattle liver between 14 to 30 USD respectively. The total losses /year were estimated as follows:

- Financial losses due to destroyed the infected sheep livers=47000 USD
- Financial losses due to destroyed the infected goats livers= 10000USD
- Financial losses due to destroyed the infected cattle's livers= 22000 USD
- The total losses of Kirkuk city = 60000 USD.

These losses did not include the unpredicted losses such as reduce of milk productive, losses of weight, fall down of wool or reduce the animal's infertility.

Dogs Hydatidosis:

Table (5) shows the results of post-mortem of 50 stray dogs, 7 dogs (14%) habitat the *Echinococcus* worm in their intestine. More than thousand worms in each dog, out of 7 dogs; one dog has severed, 2 dogs moderate and 4 dogs slight infection depend upon number of *Echinococcus* worms in their intestine, more than 1000, 200-1000, 1-10 worms respectively.

Table (5): Infection percentage of hydatidosis in stray dogs in Kirkuk city

	Male	Female	Total
Number of examined dogs	29	21	50
Number of infected dogs	5	2	7
% of infection	17.2	9.5	14

DISCUSSION AND CONCLUSION

The current study confirmed that the hydatidosis is an endemic disease in Iraq, and the percentage of infection in humans and animals is so high which indicates that the disease increases in Iraq. The high infestation of female may be due to that the female are more contact with source of infection as house

keepers, farmers and consumers of vegetables, since most female involved in the study live in rural area. Studies reported that there was relation between infection with hydatid cyst and age, since the maturation of cyst slow and the disease regard one of chronic diseases, may be continue with life unless diagnosis after the human swallow the eggs of worm and get infection (2,16). This may explain that most adult infections occur during childhood (21). The study had obtained the same results by others by which the liver has the highest percentage of damage, because single hydatid cyst goes into blood and reaches the liver (1,22,14). One of common source of infections was because of careless and poor management of slaughtering of animals in the slaughter houses and through the infected organs to dogs outside the slaughter houses. This was observed during our study and also this was reported as source of infections (15,23). An important factor that plays a key role in the spread of the disease is the fertility rate in the protoscoleces that are housed by the economic animals. It was observed that the fertility rate in the protoscoleces observed in the sheep is greater than that observed in the cystic sacs where cows and goats live (12,18). The common strain that affect the sheep in Iraq was *Echinococcus granulosus* (23), that may explain the high percentage of *Echinococcus* notice in the dogs in this study. The economic losses estimated in the current study reveals the size of problems with hydatid cysts in Kirkuk city and on other hands in Iraq, since high infestation by *Echinococcus* worm in sheep, goats and cattle due to association of aimless dogs with domestic animals. Each one infected dog can harbor more than 3000 adult *Echinococcus* worm, which have the ability to infect 3600000 individuals when there is favorable conditions (20).

This study also pointed the size of health problems in human and veterinary service.

The main conclusion of the current study is confirming that the hydatid cysts is chronic disease in Kirkuk city and representative a big health and economic problems. In order to control the disease, first of all the health and veterinary service must kills all aimless dogs in the city and rural areas, dead dogs must be buried to avoid contact and spread of infection. Secondly must watch and apply the veterinary regulation in slaughter houses with good managements.

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Initiation, growth and differentiation of *Aesculus hippocastanum* Cotyledon Calli under water stress effect

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ABSTRACT

The study had achieved callus initiation from embryogenic axes removed cotyledons isolated from sterilized seeds of *Aesculus hippocastanum*, in its growth and differentiation. The results explain the express of Murashige and Skoog (MS) medium supplemented with 0.5 mg l⁻¹ of NAA and 2.0 mg l⁻¹ of BA in callus initiation and growth by its average values of callus fresh weight which reached 10.96 g after 45 days, while the presence of 1.0 mg l⁻¹ of NAA and 2.0 mg l⁻¹ of BA encouraged callus differentiate to shoots 30 shoot in number after 45 days of growth. When we added 0.5, 1.0, 1.5 and 2.0% from polyethylene glycol PEG(6000), as a reason for water stress, the study included the positive or encouraged role for the concentration 0.5 % only in increases of fresh weight average which reached to 12.343g compared with the same medium without PEG. Furthermore found increases in proline contents with decreased in proteins, carbohydrates and nucleic acids DNA&RNA) extracted from callus with reduction in callus regeneration efficient with an increasing levels of PEG(6000) used.

Keywords: *Aesculus hippocastanum*, callus initiation, Murashige and Skoog (MS) medium

INTRODUCTION

Drought factor that affects different regions of the world, especially the arid and semi-arid agricultural areas, which is increasing annually, is very important due to the depletion of water resources, which necessitates the investigation of appropriate solutions to reduce its future damage to agriculture. Searching for the role of water stress factor on the growth and productivity of plants as well as the physiological and biochemical aspects is considered very important to understand its impact on the different stages of growth and productivity, which highlights an important step in the selection of resistant varieties for the purpose of exploitation in the field of plant breeding. In this area, several compounds were used for the purpose of water-stress testing, such as Polyethylene Glycol PEG (1), Calcium Chloride (CaCl_2) (2), Sucrose, Sorbitol and Manitol (3). Polyethylene glycol has a prominent role in water stress studies because it is a non-ionized inert material that gives great precision in determining the level of water deficit in the plant, its difficulty in permeating the plasma membranes, and its wide range of material and molecular weight and physical properties (4). The process of cellular growth has many important physiological variables that are affected by the hydrolysis resulting from some metabolic pathways of the various physiological processes of the plant to build protein, carbohydrates, nucleic acids, photosynthesis, transpiration and transport through membranes. This leads to the accumulation of some compounds that have a role in osmosis Plants (5).

Plant tissue culture is of great importance in the development of new varieties and the acquisition of drought-tolerant and drought resistant varieties such as wheat (6), pistachio (7) and thus eliminating the effect of water shortage on plant productivity (8,9). The current study aims to develop the callus from the seeds of the chestnut plant *Aesculus hippocastanum* Cotyledon removed embryonic axes and subjected to water stress using a compound of PEG 6000 (PEG) in the nutrition medium and then determine the variables that occur by measuring its growth as well as variables in the values of the biological molecules: prolines, carbohydrates, proteins and nucleic acids.

MATERIALS AND METHODS

Initiation and maintenance of the cotyledon calli cultivars removed sterile embryonic axes:

Seeds of chestnut (*Aesculus hippocastanum*) were brought from local markets of Mosul city/ Iraq. The outer hard shells and the embryonic axes were removed, then sterilized by dipping in Ethyle alcohol solution 96% for 2 min, followed by dipping in Sodium Hypochlorite commercial solution (NaOCl) called (Fas) that is manufactured by Babel wet weights were varied according to the variation of the other used nutrition media. Therefore, the

Company for soap and detergent manufacturing, Baghdad) with concentration 6.2%, and diluted in a ration 1:2 NaOCl : sterilized distilled water, for 10 min, with continuous shaking. After that, they were washed by sterilized distilled water three times / min. cotyledons were cut into a number of small pieces, nearly 0.5 gm and put on a 30 ml of MS medium (10), supplied by different concentrations of NAA, BA (1.0, 2.0, 0.5 mg l^{-1}) for each separately for one time, and mixed for another in order initiate and develop the callus and its growth. Furthermore, cotyledons were cultivated on MS medium, that contains no growth regulators for comparative purposes. Samples were kept in an incubator at temperature ($22 \pm 2^\circ\text{C}$), supplied by light source with a daily frequency 16 hrs light/ 8hrs darkness (1500 lux).

Measuring wet weight:

Wet weight of calli was estimated after 4 days by taking 0.3 gm from the initiated callus on MS medium supplied by different concentrations of NAA and BA (0.5, 1.0, 2.0 mg l^{-1}). Also, for the wet weight of growing callus on same media supplied by different concentrations of PEG compound. The best medium were considered (MS with 0.5 mg l^{-1} NAA and 2.0 mg l^{-1} BA) for comparison one time, and with addition of (0.5, 1.0, 1.5, and 2.0%) of PEG another time to measure the variables in the following biological molecules:

1-amount of proline: were estimated according to Bates *et. al.* (11) method.

2- amount of carbohydrates: were estimated by using Herbert *et. al.* (12) method.

3- amount of protein: method of Lowry *et. al.* (13) was followed to evaluate protein.

4- amount of total nucleic acids: the standard method was followed (14), while the method for evaluating the content of RNA and DNA in callus cells was according to Giles and Mayer (15).

RESULTS AND DISCUSSION

Production and growth of chestnut (*Aesculus hippocastanum*) cultivars:

Results showed superiority of MS solid medium supplied by adding 0.5 mg l^{-1} NAA as well as by adding 2.0 mg l^{-1} BA in callus initiation from cotyledons of chestnut (*Aesculus hippocastanum*) removed embryonic axes during 25 days, where the callus had been distinguished by green color and its fragile texture, followed by the other concentrations of NAA and BA (table 1). However, MS solid media alone had failed in initiation of callus, moreover, it was followed by increase in wet weight of the growing callus on the best initiating medium to reach 1.096 gm after 45 days (table 2). Values of

initiation and development of callus depends strongly on the existing percentages of oxynes and

cytoxines in the nutrition media and their roles of balancing to stimulate successive divisions of plant

tissues (16).

Table (1): Initiation and development of *Aesculus hippocastanum* Cotyledon Calli after 25 days in MS solid medium supplied by different concentrations of NAA and BA

Conc. Mg/l BA NAA	Degree of initiation			
	0	0.5	1.0	2.0
0	Zero	50	85	80
0.5	30	50	70	95
1.0	25	65	60	80
2.0	30	70	Zero	80

Each value represents the average of 5 records

Table (2): Ratios of wet weight of *Aesculus hippocastanum* Cotyledon Calli after 45 days in MS solid medium supplied by different concentrations of NAA and BA

Conc. Mg/l BA NAA	Ratios of wet weight (gm)			
	0	0.5	1.0	2.0
0	0.730	1.667	9.025	4.432
0.5	1.511	2.011	3.064	10.960
1.0	0.930	2.860	3.745	3.275
2.0	1.123	4.545	0.422	4.051

Each value represents the average of 5 records

The effect of poly ethylene glycol (PEG) (6000):

A-on wet weight of callus: results showed that the addition of 0.5% PEG to MS standard solid medium had encouraged callus growth. This can be indicated by the values of wet weight that were 12.343 gm compared with 9.530 gm on MS medium (comparison). Callus was distinguished by its greenish yellow color and its fragile texture (figure 1-B, table 3), while concentrations 0.1, 1.5 and 2.0% PEG had discouraged the ratios of callus wet weight after 30 days, and the callus had distinguished by its black color and dissociated texture (figure 1-C, table 2). These results could be due to the fact that little concentration of PEG 0.5 % to simulate callus wet weight due to its ability to absorb large amount of water and nutrition available

in the nutrition medium to keep water stress balance for the medium of tissue cultivating medium of *Aesculus hippocastanum* Cotyledon (17). In addition, the reduce in wet weight ratios of callus in the presence of high concentrations of PEG on the nutrition media may be according to reduction of water ability and dissolved nutritive materials which require cellular reorganization for its osmotic stress to adapt with water stress that was caused by PEG in the nutrition medium which in turn affect negatively on the tissue division and growth (18). Also, a reduction in rice callus wet weight ratios at growth on a medium that contains high concentrations of PEG, while the low concentrations of PEG caused an increase in callus weight (9,19).

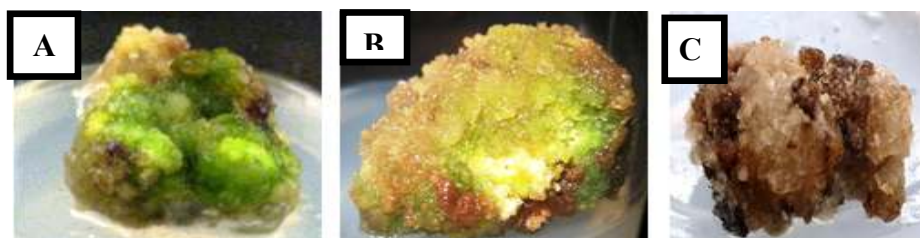


Figure (1): the growing *Aesculus hippocastanum* Cotyledon Calli after 45 days on media: A- MS standard (contains 0.5 mg l⁻¹ NAA and 2.0 mg l⁻¹ BA) B- MS standard + 0.5% PEG. C- MS standard + 2.0% PEG

Table (3): the effects of PEG on ratios of callus wet weight derived from *Aesculus hippocastanum* seed cotyledons and contents of proline, carbohydrates, proteins and nucleic acids after 30 days growth

PEG conc. (%)	Wet weight (gm)	Proline (mg/gm)	Carbohydrates (mg/gm)	Proteins (mg/gm)	DNA (mg/ gm)	RNA (mg/ gm)
*comparative coefficient	9.530	6.211	355.20	0.951	12.213	100.704
0.5	12.343	8.356	325.14	0.781	12.211	100.693
1.0	5.411	10.920	280.20	0.632	11.030	88.241
1.5	2.501	13.511	141.50	0.431	8.212	70.923
2.0	1.050	16.254	130.30	0.213	2.310	20.791

Each value represents average of 3 records

*comparative coefficient represents standard media (MS contains 0.5 mg l⁻¹ NAA and 2.0 mg l⁻¹ BA without PEG)

B- callus content of proline: results revealed an increase in proline amounts according to increase used PEG concentrations, by which it reached 16.254 mg/gm in growing callus on standard nutrition medium supplied by adding 2.0% PEG. This may be due to the proline osmotic and protective role, where it accumulates in the tissues of high plants that suffer from osmosis through induction of new enzymes, or rebuilding and stopping damage (20). Thus, proline can be classified as one of the obligatory amino acids to behave in certain physiological cases to create a kind of balance between the gap and cytoplasm (Hassan *et. al*, 2004), and then to act as protective factor for cells at growth media (21).

C- callus content of carbohydrates: it is clear from the results obtained the reduction in carbohydrates concentrations in the samples of *Aesculus hippocastanum* seed cotyledons when PEG concentrations increased. The highest ratio of carbohydrate concentration was 355.2 mg/gm on MS solid standard media, where the lowest concentration was when adding 2.0 % PEG to the standard media to be 130.3 mg/gm (table 3). The reduction in callus content of carbohydrates with the increasing concentration of PEG in the nutrition media is caused by the great energy made by the callus cells to resist ionic disturbance inside cells by increasing the breathing and then metabolism of carbohydrates occurs (9).

D- callus content of protein: according to the obtained results, the addition of PEG in concentration 1.0, 1.5, 2.0 % to the standard media had lead to decrease in callus content of protein after 30 days growth period, and were 0.632, 0.431, 0.213 mg/gm respectively (table 3). The decrease in cell protein contents is caused by the reduction of tissue ability during water stress to involve amino acids in proteins, and results obtained from a study conducted on sunflower plant under water stress conditions found that the increase in amino acid contents may be due to the fast protein degradation (22), same as for rice plants, that caused a disturbance in the biological pathway for building proteins (23).

E- callus content of total nucleic acids: results showed decrease in callus content of DNA and RNA when suffering from water stress after 30 days period of cultivation in high concentrations of PEG, while approximate values were recorded from values of comparison samples when adding 0.5% PEG (table 3). Previous studies had proved that water stress causes defect in cell contents of nucleic acids and some enzymes such as RNase and others (24). Furthermore, water stress was found to cause defect in creation of DNA and RNA, and that the content of nucleic acids in some species of corn increase in lower concentrations while decrease in higher ones (25), and the higher concentrations of PEG can cause reduction in water content which in turn leads to stopping the progress of cellular cycle through breaking down the two mytotic division phases G1 and G2, before and after forming DNA respectively, which means reduction in formation of nucleic acids or speeding up their breakdown (26,27).

It is clear from the above, that cells exposed to water stress can maintain their fullness by changing content concentrations of proline, carbohydrates, proteins and nucleoid acids, and other molecules to reduce water stress into a level less than outside surrounding.

F- callus differentiation: results revealed differentiation of 30 vegetative branches of *Aesculus hippocastanum* seed cotyledons removed embryogenic axes after 45 days period of growth on top of MS media contains 1.0 mg l⁻¹ NAA and 2.0 mg l⁻¹ BA (figure 2-A). the presence of PEG with all used concentrations had lead to discourage the process of differentiation (figure 2-B). these results were in agreement with that obtained by (28), when no differentiation occurred for wheat callus exposed to water stress when transferring to a differentiation medium, also it was found that an increase in PEG concentration caused decrease in differentiation ratios in both rice callus and sugar cane callus (17).

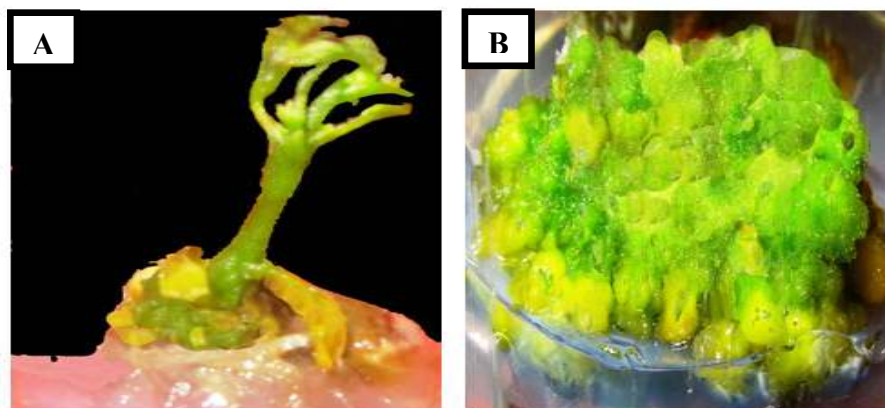


Figure (2): differentiation of *Aesculus hippocastanum* seed cotyledon callus removed embryonic axes after 70 days of growth on media: A- MS (MS contains 1.0 mg l^{-1} NAA and 2.0 mg l^{-1} BA). B- MS +0.5 % PEG

It can be concluded that *in vitro* plant cultivation technique will help researchers to go in deep among the related issues of water stress in order to have fast and effective solution to this problem Which began to worsen and the problems of desertification and water scarcity, as well as the ability to know all changes occur inside plant tissues exposed to water stress.

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Removal of chromium from aqueous solution using (reduction/sorption) bed from iron waste and nutshells

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ABSTRACT

In this research removal of chromium from an aqueous solution by Multilayer-Mixed (reduction/sorption) bed via scrap iron as zero valent iron and charred nutshell(walnut shell) as adsorber were studied. Both batch and continuous system were conducted , the batch system reveals that the adsorption process was of favorable type . Three models were used to describe the adsorption process. Freundlich model is matching well to the data with ($R^2=0.975$). In the continuous system , two sets of experimental were considered, the first set (Multilayer bed) conducted by fixing the characteristics of the scrap iron bed (bed height 0.2 cm, particle size 500 μm) and varying the walnut shell parameters, the optimum value of this set recorded as (bed height 2.5 cm, particle size 250 μm). While for the second set of experimental after fixing the optimum values of the walnut shell bed and varying the scrap iron bed characteristics ,the optimum values was (height 1cm, particle size 500 μm as filling). Also, the Mixed bed configuration were studied by conducting the optimum conditions for the previous experiment. it found that at any certain time the mixed layer offers the maximum efficiency of removal rather than the multi-layer bed.

Keywords: Scrap iron; Chromium; adsorption; walnut shells

INTRODUCTION

Environment pollution is a worldwide problem and have a great potential and influence to human health (1,2). Pollution reaches it is most serious proportions in the densely settled urban-industrial centers of the more developed countries (3). Heavy metals can be found in dissolved form, particulate and colloidal phases, the present of metals in water bodies can be caused by either anthropogenic origin (e.g. waste water disposal) or natural origin (e.g. leaching of ore deposits) (4,5). Chromium is a metal that have extremely importance and variety uses in industries like: textile dying, tanneries, metallurgy, chemical and refractory industries, wood preserving, preparation of chromate compounds, metal electroplating. Hexavalent Chromium (Cr^{+6}) compounds show a high environmental mobility and have toxic effects in most living organisms (6). Trivalent Chromium (Cr^{+3}) is undesired metals that have adverse effects on human health and can accumulate in the food chain, that will be cause numerous diseases. Many industries use trivalent chromium Cr^{+3} and disposed it from their effluent, such as electroplating, tanning and leather. In waste water the maximum level for Cr^{+3} is 5mg/L (7).

MATERIALS AND METHODS

This study made to assess the use of scrap iron fillings (as a zero valent iron) to continuously reduced of Cr^{+6} to Cr^{+3} and subsequent adsorption of Cr^{+3} onto charred walnut shells. The effect of different factors and conditions on treatment process such as bed height and particle size of scrap iron and charred walnut shell had been studied.

Zero valent iron fillings:

Scrap iron filings was used in this study as a zero valent iron (ZVI, with oxidation number of zero, Fe^0), type SELKA GmbH, Hamburg, Code 46 12.12, Germany.

Preparation of adsorbent:

Walnut shells were collected from local markets and washed with deionized water for several times to remove impurities and dirt. Wetted Walnut shells were dried at 100 °C as reported by (8). The dried Walnut shells were grinded and then charred in a laboratory furnace set of (600 °C) for (1 hr) (8). The resulted charred walnut shells were sieved to (250 μm , 600 μm , 1mm) size with using (Sieves type, S/N: 0307314, Body size (200 \times 500) mm, made in Germany) these different size to study the influence on the adsorption process by changing particle size.

Contaminant:

The stock solution of Chromium with concentration of (1000)ppm (mg/L) was set through dissolve (5.6584)g of ($\text{K}_2\text{Cr}_2\text{O}_7$) potassium dichromate in (1L) volume of de-ionized water. Prepared stock solution was diluted finally to get the desired concentration of chromium ions, by using equation (1) (9).

$$W = C * V * \frac{M_{wt}}{At_{wt}} \dots\dots (1)$$

Where:

W : the metal mass of $\text{K}_2\text{Cr}_2\text{O}_7$ (mg)

C : the required concentration (mg/L)

V : solution volume in (L)

At_{wt} : the atomic weight, Cr= 51.99 gm/mole

M_{wt} : the molecular weight, for potassium dichromate= 294.1846 gm/mole.

Batch Experiment:

Batch experiment was conducted by using constant mass of one of the two waste materials with different masses for the second material (e.g add 0.5 gm of scrap iron to (0.2, 0.4, 0.6, 0.8, 1, 1.2, 1.4, 1.6, 1.8, 2 gm) of Char prepared from Walnut shells and then use the optimum dose of the Char with different masses of scrap iron (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1gm). Stock solution was diluted to obtain the needed concentration (25 mg/L) of chromium ion.

Samples of (100ml) of the known concentration-solution were placed in flasks (conical), with accurate masses of walnuts waste adsorbent and scrap iron. pH=7 (was measured), temperature = 25°C. After that 10 flasks were put in rotary shaker (No.SM-25, Edmund, Made in Germany) with speed of agitation (100 rpm) for 24 hrs.

Continuous System:

Glass tube for length (35 cm) and inner diameter (4.5cm), was used with distributor to insure a good distribution of the solution over the bed. Glass mesh also was used for preventing the materials losses. When the run is on, the solution of Chromium -with known concentration- was passed through the bed. (40L) volume plastic container was used for containing the stock solution. A flow meter was used for measuring the flow rate. Electrical pump (HP-9600D) used to pump the Chromium solution from the feed solution to the bed. The bed is packed with scrap iron and charred walnut shells in two systems (separated, mixed).

RESULTS AND DISCUSSION

Batch system:

Experimental data for batch experiment was fitted to Langmuir and Freundlich isotherm, founded that Freundlich isotherm was gave the pest fitting according to R^2 value which equal to (0.975). (Figure 1 and 2).

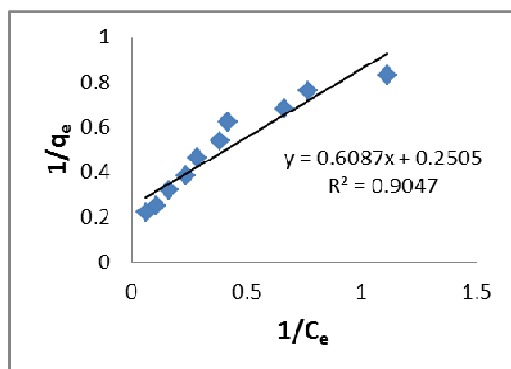


Figure (1) : Langmuir model

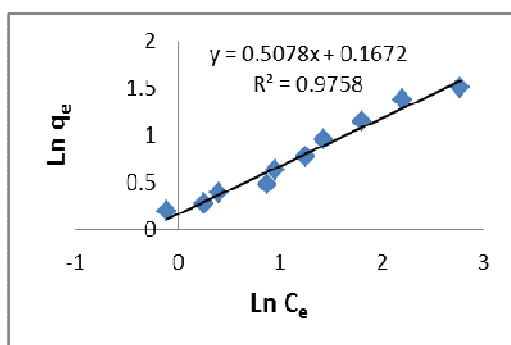


Figure (2): Freundlich model

Continuous system:

1-Effect of bed height of charred walnut adsorbent: The effect of varying the bed height of the charred walnut shell adsorbent (0.5, 1, 1.5, 2, 2.5 cm) were conducted by fixing these parameters (bed height and particle size of scrap iron are (0.2) cm , and (500) μm respectively. Particle size of charred walnut shell adsorbent 600 μm . Initial concentration C_0 25(mg/L), temperature 25 $^{\circ}\text{C}$. pH 5.8. Flow rate 0.5 L/hr) . The experimental breakthrough curves are presented in (figure 3) as C_d/C_0 versus time.

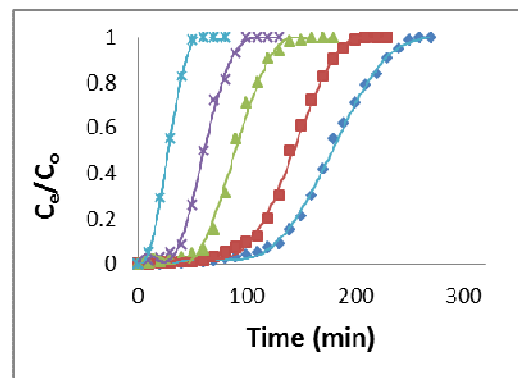


Figure (3): Breakthrough curves for different bed height of charred walnut shells adsorbent

From this figure it is clear that when the height of bed increases, the breakpoint (in the breakthrough curve) increase. The reason is, as bed height is at great the ratio of adsorbate concentration in the effluent will be increasing slower than for a smaller bed height. Moreover, the time for saturation is increase. Greater bed heights correspond to greater mass of an adsorbent, as a result, a greater capacity for bed (10).

Increasing the bed height will increase the contact time of the adsorbate with the bed, thus improving the adsorbate removal efficiency. As the bed height increase, the adsorption capacity will increase too, corresponds to extra spaces on charred walnut shell active sites will available for the adsorbate molecule for adsorpting on these empty active sites (11).

2-Effect of particle size of charred walnut adsorbent: The effect of using various particle sizes (1mm, 600 μm , 250 μm) of charred walnut shell on the breakthrough curves were investigated with fixed the other parameters. Bed height of charred walnut shell 2.5 cm. Initial concentration C_0 25 mg/L. Temperature 25 $^{\circ}\text{C}$. pH 5.8. Flow rate 0.5 L/hr). Experimental breakthrough curves are shown in figure (4).

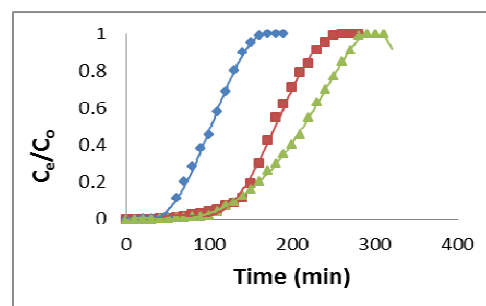


Figure (4): Breakthrough curves for different particle size of charred walnut shells adsorbent

The breakthrough curves indicated that the time required for reaching breakpoint increase when particle size decrease. This occur because that surface area available for adsorption increases with decreases the particle size, that mean more sites for adsorption and the adsorption capacity increase Figure (4) reveals that, as the particle size decrease, it will increase the time for saturation, and the film thickness that can be found around the adsorbent particle is increase, and inside the pore structure the length of the path also increases. Therefore, the process kinetics is low; due to the adsorbate molecule will need more time to reach the active site on adsorbent, since the larger diffusion path inside the pores (12).

3-Effect of bed height of scrap iron: Three experiments were conducted for different bed height of scrap iron (0.2, 0.5, 1 cm) to examine breakthrough curves, with optimum conditions from the previous experiments. Experimental breakthrough curves are plotted in figure (5).

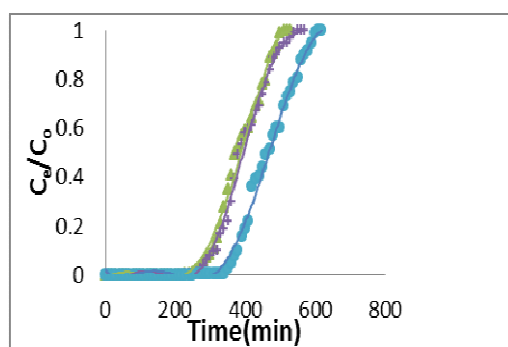


Figure (5): Breakthrough curves for different bed height of scrap iron

As it was observed from the previous experiment, and from figure (5) as the bed height of scrap iron increases, the breakpoint increase. The sorption capacity increase also as the bed height increased. This can be attributed to the increase in specific surface of the sorbent (more binding sites) this will cause a delay in the breakthrough of the pollutant In addition, the time required for saturation and breakthrough are increased for long columns, as reported by (13).

4- Effect of shape and size of scrap iron: Effect of using different particle shape (spiral, fillings) with dimensions (length 3-7 mm and diameter 3-3.5 mm) for spiral, and (500 μ m) for fillings scrap iron, on the breakthrough curves were studied by keeping the other parameters constant. Experimental breakthrough curves presented in figure (6).

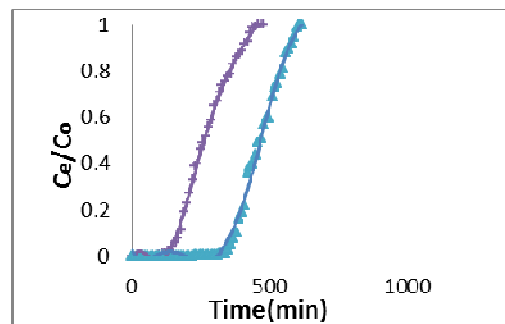


Figure (6): Breakthrough curves for different particle size of scrap iron

The previous figure indicated that when particle size of scrap iron decrease, the breakpoint would be increase, this attributed to increasing the surface area that needed for reduction, when the particle size decreases that mean additional sites would be available for solute and the reduction capacity increase (13).

CONCLUSION

Two waste materials offering a good recyclable alternative to be as a cheap reductant/adsorbent for removal of Hexavalent, Trivalent Chromium from aqueous solution.

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Visualization and analysis of Tumor protein P53 by VMD

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ABSTRACT

It is well known, that proteins are large, complex molecules that perform many essential roles in the body. They are either structural or functional; they do most of the work in cells, and play important role in regulation of the body's tissues and organs. In our study we used VMD software to analysis tumor proteins P53. VMD (Visual Molecular Dynamics) is a molecular visualization and analysis program designed for biological systems such as proteins, nucleic acids, lipid layer assemblies, etc. The technique has several step-by-step examples of some of VMD's most popular features, including visualizing molecules in three dimensions with different drawing, coloring methods, and analyzing both sequence and structure data for proteins.

Keywords: VMD, P53, analysis, protein

INTRODUCTION

Human p53 is a nuclear phosphoprotein of MW 53 kDa, encoded by a 20-Kb gene containing 11 exons and 10 introns (Lamb and Crawford, 1986) which is located on the small arm of chromosome 17 (1), p53 contains 393 amino acids consisting of three functional domains, i.e. an N-terminal activation domain, DNA binding domain and C-terminal tetramerization domain. P53 protein was first identified in 1979 and identified as a transformation- related protein (2) and a cellular protein which accumulates in the nuclei of cancer cells and binds tightly to the simian virus 40 (SV40) large T antigen (3). p53 plays essential role as tumor suppressor after obtaining data from the first p53 knockout mice, meaning that mutations of in a mouse lacking p53 could not be suppressed (4). Commonly identified as a tumor suppressor, and the p53 gene became possibly the most common site for genetic alterations in human cancers (5). p53 is known to have a critical role in almost all types of human cancers, and the mutation or loss of the p53 gene can be identified in more than 50% of all human cancer cases all over the world.

To better understand the p53 protein, it is crucial to visualize the protein structure meaning displaying, animate and showing three dimensional structure of the protein. In this study, p53 protein was visualized by using Visual Molecular Dynamics (VMD).

It is developed by the Theoretical and Computational Biophysics Group at the University of Illinois at Urbana-Champaign. Among molecular graphics programs, VMD is unique in its ability to efficiently operate on multi-gigabyte molecular dynamics trajectories, its interoperability with a large number of molecular dynamics simulation packages, and its integration of structure and sequence information (6). In order to visualize the protein molecule, protein file from protein data bank (PDB) must be obtained first.

The Protein Data Bank (PDB) is a repository for the three-dimensional structural data of large biological molecules, such as proteins and nucleic acids. The data, typically obtained by X-ray crystallography or NMR spectroscopy and submitted by biologists and biochemists from around the world, are freely accessible on the Internet via the websites of its member organizations.

MATERIALS AND METHODS

Materials used for this study were few and simple, as the study is based on bioinformatics. However, the methodology was quite long. A conventional personal computer and VMD software version 1.9.1 was used, at the same time this website <http://www.rcsb.org/> was used from which the file of p53 protein was downloaded.

The VMD software version 1.9.1 installed in a conventional personal computer, and then it was

opened. There are seven toolbars in the VMD main window including File, Molecule, Graphics, Display, Mouse, Extensions and Help. In our study, we worked on, Graphics, Display, Mouse and Extensions. Firstly, File tool bar was clicked then from which some other options were opened one of them was New Molecule. The New Molecule option was clicked then another window was opened for browsing the molecule file. The P53 file was browsed then loaded on the VMD software. Afterwards, in order to provide more comprehensive illustrations, the molecule was manipulated by using the four above toolbars.

1-Graphics: From graphics, two options were selected to visualize the p53 including Representations and Colors. After that, in the Graphical representations there was Draw style in which we selected Drawing method and coloring method in drawing method some options were tested and for the coloring method some options were tested.

To display only parts of the molecule of interest, we could specify their selection in the graphical representations window.

To display only parts of the molecule of interest, we could specify their selection in the Graphical Representations window. In our molecule p53, the protein molecule is composed of beta sheet and helix bound to DNA double strand. In order to see the molecule without helices and β -sheets, we just typed the following in Selected Atoms: (not helix) and (not betasheet). Then pressing the Apply button or hit the Enter/return key.

2- Creating multiple representations: The button Create Rep in the Graphical Representations window allows us to create multiple representations. For example, for our initial representation, in Selected Atoms type protein, we set the Drawing Method to New Cartoon and the Coloring Method to Structure. Afterwards, by pressing the Create Rep button. A new representation was created. We modified the new representation to get VDW as the Drawing Method, Res Type as the Coloring Method, and rename LYS as the current selection.

3-Mouse: Mouse is one of the toolbars which very essential in VMD software, after clicking on Mouse some other functions will be opened to allow users manipulate the selected molecule for example Rotate mode, by clicking on it we could rotate the molecule to any direction we wanted. Translate mode is another option within the Mouse option; we could drag the molecule to any position in the VMD display scale. For zooming in and out we could click on scale mode, this option enable users to zoom in the molecule to a size that could be clearly seen.

4- Display: For displaying our molecule we need to use Display option in the VMD main window, in our study we worked on the Axes, Background and Stage. We could switch on and off the Axes, which disappears as we switched off. We then could move the Axes to different locations around the molecule within the Open GL Display window by selecting lower left, Lower right, Upper left and Upper right of the molecule appeared within the window. There are two options within the background option, the background of the molecule is originally solid color but we could change it to light blue color by selecting Gradient option within the Background option. Stage is another option which we worked on, this option enables users to give a stage to the top, bottom, left, right or behind the 3D structure of our molecule P53.

5- Extension: We worked on another useful option which is Extensions; in this option we selected the sequence viewer option then VMD sequence viewer. The sequence viewer is used to list the residue sequences of proteins and the base sequences of nucleic acids, and to select residues is color-coded in columns and obtained from STRIDE. The "B-value" column shows the B-value field (Temperature factor) often provided in PDB

files. The structure column shows secondary structure, where each letter corresponds to a secondary structure, Color-coded protein structure information is displayed for amino-acid residues, and B-factor information is displayed for all residues. In this section, "residues" refers both to amino acid residues in proteins, and to nucleotide bases with associated backbone in DNA and RNA molecules. Secondary Structure codes used by STRIDE, are illustrated in table (1). We choose another option of analysis is Ramachandran plot. Ramachandran plot create 3D histogram parameter the result will come on mean page OpenGL. The main window contains a Ramachandran graph, with phi and psi running along the horizontal and vertical axis, respectively, from -180 to 180 degrees. The most allowed region of Ramachandran space is colored blue; partially allowed regions are colored green.

In the extension of the VMD main window from analysis go to contact map, choose calculate parameter then calc. res-res dist. The Contact Map plug in provides an easy-to-use interface for viewing residue-residue contacts between two sets of selected atoms from molecules loaded into VMD.

Table (1): Secondary structure codes

Code of stride	Means of secondary structure codes used by STRIDE
T	Turn
E	Extended conformation (β -sheets)
B	Isolated bridge
H	Alpha helix
G	3–10 helix
I	Pi helix
C	Coil

RESULTS AND DISCUSSION

After installation of the VMD version 1.9.1 the program ready for use by clicking on window start then selecting the VMD program, after selection of the program three windows were opened. These three windows could enable us to visualize our molecule of interest p53. Firstly, we uploaded the

protein molecule file which was already saved in our PC drive. After clicking on File from the VMD Main window we then clicked on new molecule then another window was opened which asked for Browsing the molecule after browsing the P53 file then we clicked on upload button. Afterwards, the molecule protein was appeared in the VMD 1.9.1 OpenGL Display (figure 1).

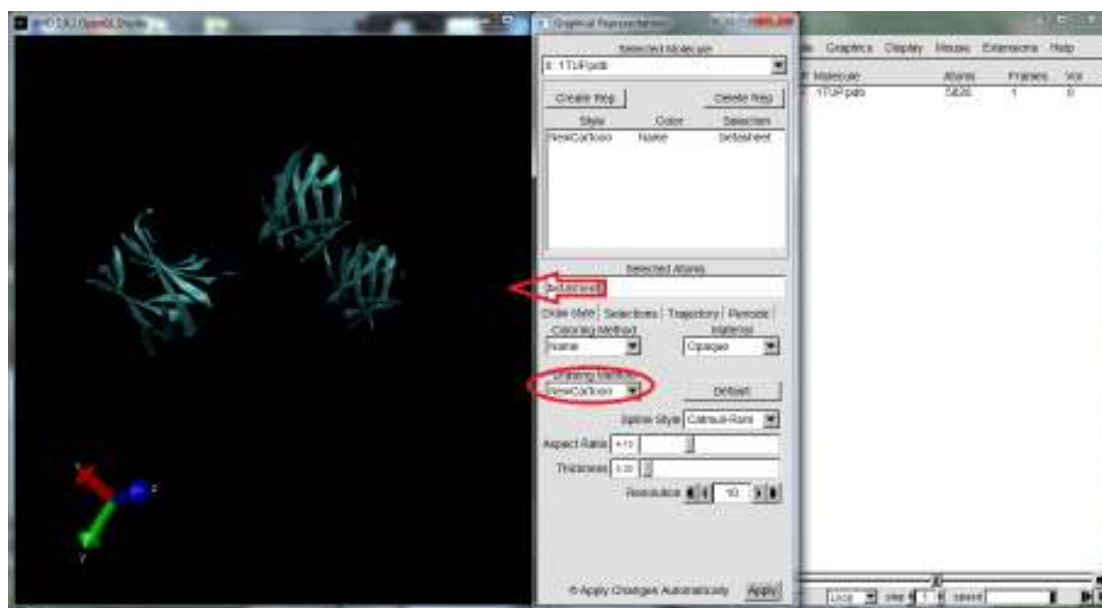


Figure (3): the protein selected atoms (only betasheets) with the New cartoon drawing method

It gives a simplified representation of a protein based on its secondary structure. Helices are drawn as coiled ribbons, β -sheets as solid, flat arrows and all other structures as a tube. This is probably the most popular drawing method to view the overall architecture of a protein.

We also showed the protein atoms except helix and beta sheet by using Boolean operator when we writing a section (figure 4).

We have also shown the water molecules in p53 (figure 5).

Thus it could be said that the Graphics option within the VMD program is very important tool for representing a chosen molecule, in different drawing method and in separate forms. This could help the users to see the protein constituents which can be used for many purposes in proteomics and in terms of therapeutics for designing drugs.

In the coloring method, choose a drawing method that shows individual atoms: each atom will have a different color, i.e. O is red, N is blue, C is cyan and S is yellow (figure 6).

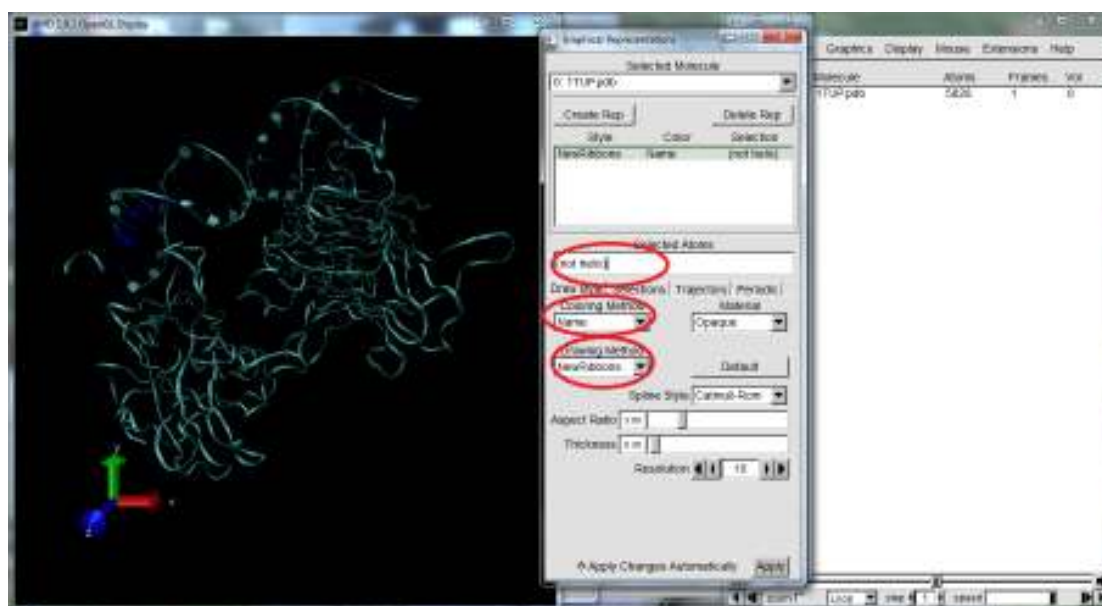


Figure (4): The protein atoms of p53 without helix and beta sheet structures

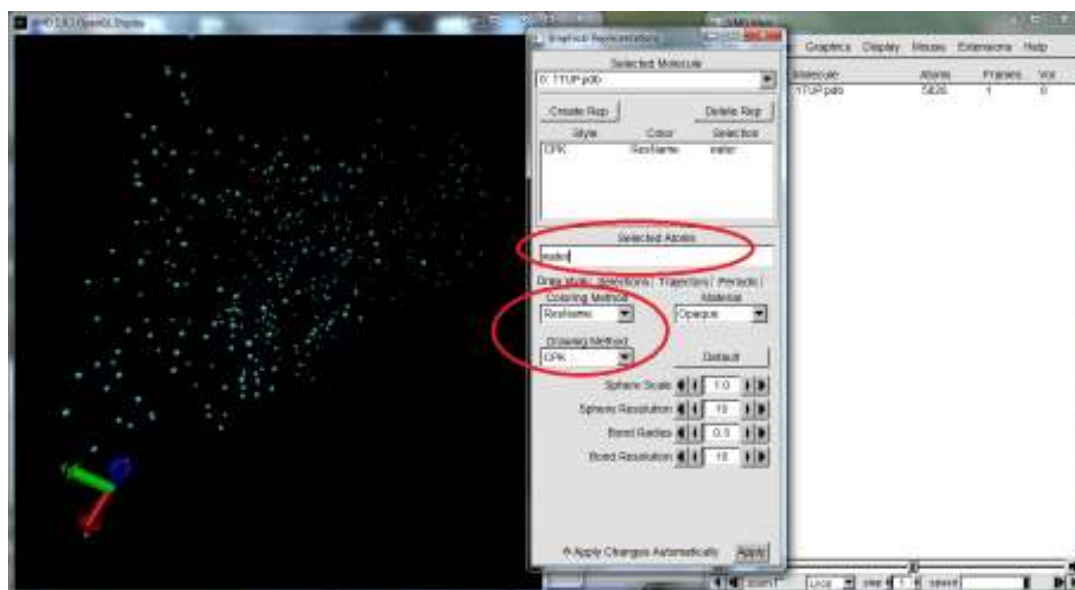


Figure (5): The water molecules in the P53 protein molecule

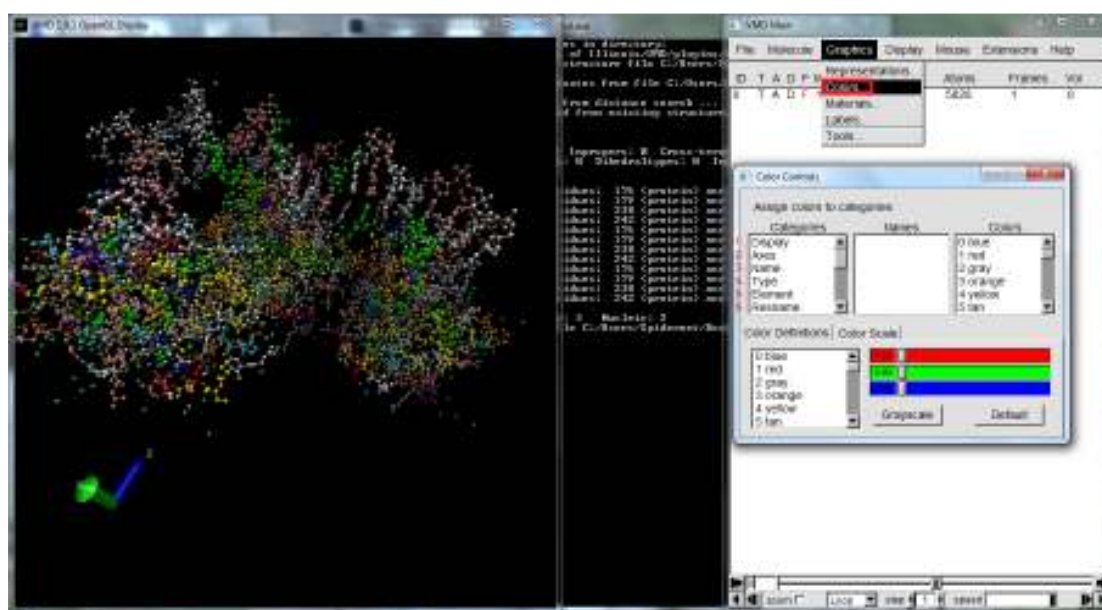


Figure (6): The selected protein with different colors

This means that once we select green color to gray, then all glycine within the p53 protein will be appeared in green color. We could also change the color of the bonds, chains, axes...etc (7).

Mouse:

Mouse is another option in VMD which can be used for some crucial purposes, it helps users to give more comprehensive illustration. As the protein molecule is appeared in the VMD OpenGL Display, it can be rotated 360 degrees around the molecule

itself. This gives a better three dimensional visualization of the molecule. We could label our molecule constituents separately including Atoms, Bonds, Angles and Dihedrals. The information about the labeled part is appeared in the Program files x86 (figure 7). Another useful option is the Mouse Center menu item. It allows you to specify the point around which rotations are done (8).

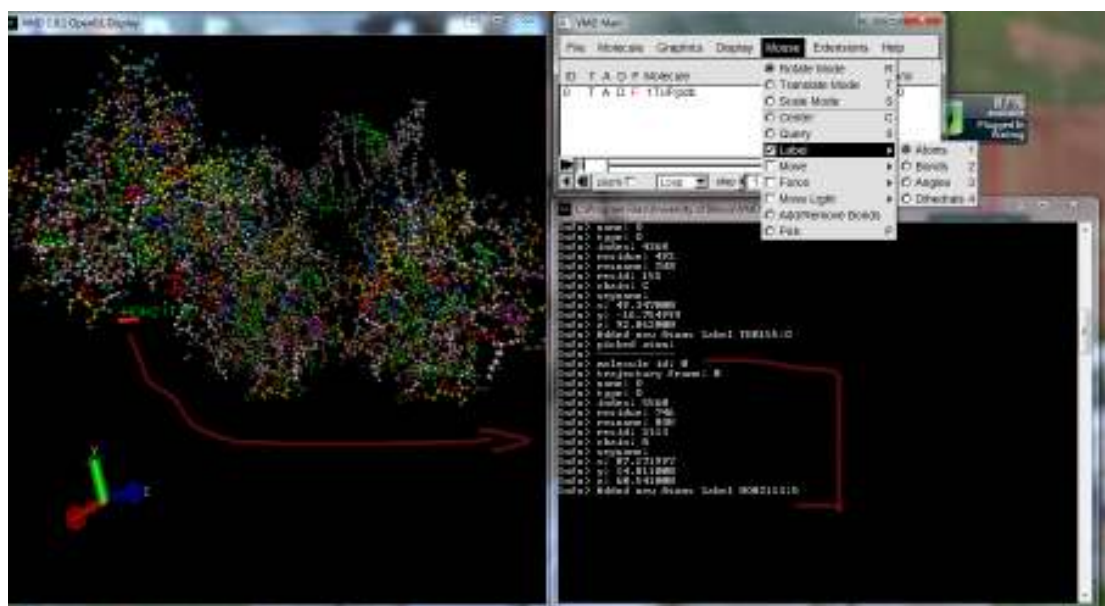


Figure (7): p53 in which an atom is labeled and the information about this atom is appeared in the program files x86 as pointed in red color

Extension:

In extension chosen (sequence viewer, Ramachandran plot, and contact map). When residues are selected in the main VMD window, the corresponding residue is highlighted in the sequence list in this form (9,10). Color-coded protein structure information is displayed for amino-acid residues, and B-factor information is displayed for

all residues. In this section, "residues" refers both to amino acid residues in proteins when selected amino-acid appear yellow color from VMD 1.9.1 display (figure 8). another option is Ramachandran plot, used for a selected molecule and create 3D histogram parameter (figure 9). Contact map viewing residue-residue contacts between two sets of selected atoms from molecules loaded into VMD (figure 10).

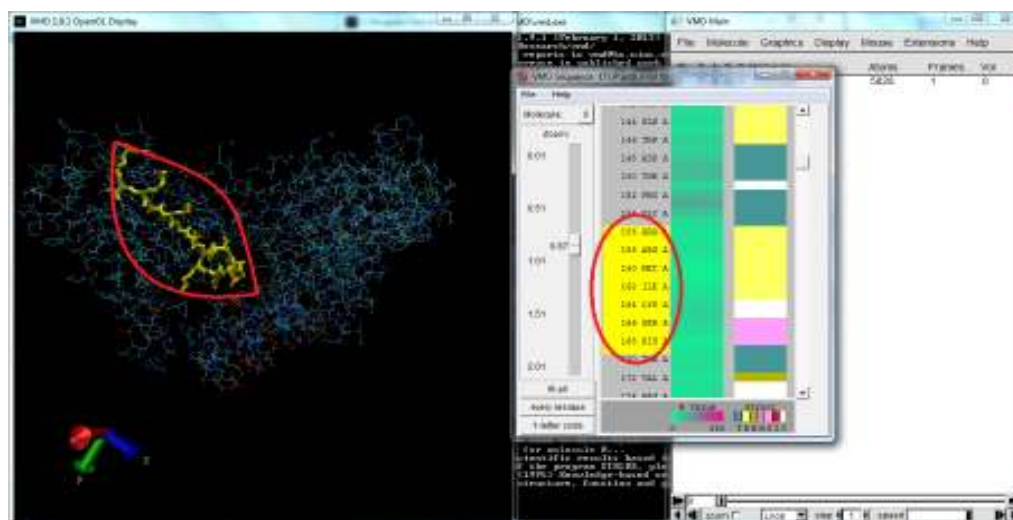


Figure (8): The sequence window

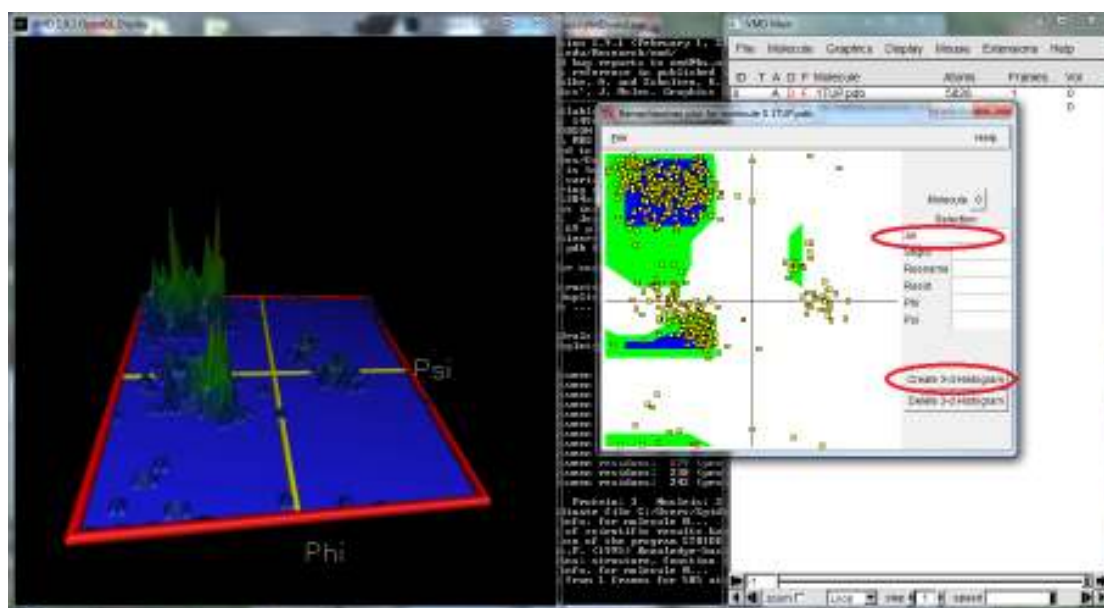


Figure (9): Ramachandran plot and histogram showed for protein

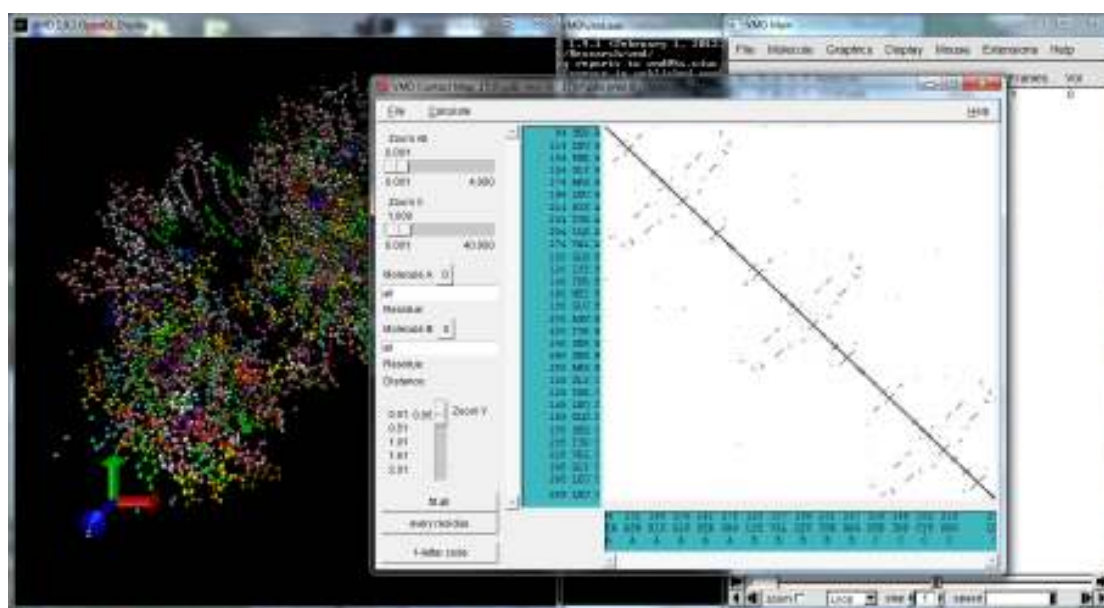


Figure (10): Residue- residue contact for the same protein

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