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FORWARD

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Today, we announce new two issues of our journal, that are the second and the third issues from the sixteen volume of IJST, September, 2021.

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

TABLE OF CONTENTS

** Articles in this issue are listed below according to alphabetical order*

Detection of objectionable and non-objectionable microorganisms in contaminated paracetamol dosage forms

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ABSTRACT

Paracetamol dosage forms are types of over-the-counter (OTC) medicine, non-prescription medicine. Similar to other drugs, they are potentially susceptible to contamination by microorganisms during production.
The purposes of this study were to investigate the occurrence of microorganisms contamination of paracetamol tablets, syrups and suppositories according to USP chapter 61, and to test the strength of the preservative ingredients according to the antimicrobial effectiveness test of USP chapter 51. Sixty samples of commercially available preparation forms were collected randomly from pharmacies in Amman city. Samples of different preparations were included using Total Aerobic Microbial Count (TAMC) and Total Yeast and Mold Count (TYMC) tests by streaking method on the surface of different media to investigate bioburden. The isolates were identified using biochemical tests and Remel RapID ™ systems. Six samples were used in the Anti-microbial Effectiveness Test (AET), three samples have been found loaded with microorganisms and the other three samples were considered as control. These samples were inoculated with specific microorganisms mentioned in USP chapter 51. Samples were investigated for the effectiveness during 28 days. Nineteenth out of 60 tested samples were found to have varies degrees of microbial contamination. The highest bacterial count ($1x10^5$ cfu/g) presented in tablet sample A25 and suppository sample C15. While other samples showed viable count ranged between (50 cfu/g or g) and ($1.5x10^2$ cfu/ml or g). Microorganisms isolated were *S. aureus, S. epidermis*, *staphylococcus spp.*, *bacillus spp.* and *Enterobacteriaceae*. The isolated yeasts were namely *Candida spp.,* and filamentous mold: *Penicillium, cephalosporum, and mucor.* The AET showed 2-3 log reduction in the samples does not possess microbicidal reduce the total number of challenged microorganisms, compared with contaminated samples which showed 1 log reduction or no reduction with visual changes in color and fungal surface growth. Challenge test for pure paracetamol did not show any anti-microbial activity when tested against *E.coli, S.aureus* and *P.aurgeinosa* and no inhibition zone at all. Some dosage forms of paracetamol showed contamination by microorganisms. For the bioburden test; presence of objectionable microorganisms like *Staph spp., Enterobacteriace* and *Candida spp.*, which caused the rejection of products. Furthermore, for the other microbes, it may become objectionable since they exceed the acceptable limits of microbial count. Samples which did not pass the test; preservative types and concentration were investigated to ensure their effects along their shelf lives.

Keywords: Paracetamol, dosage forms, contamination, bacteria, fungi, Total Aerobic Microbial Count (TAMC), Total Yeast and Mold Count (TYMC) test.

INTRODUCTION

Paracetamol (acetaminophen) is an analgesic and anti-pyretic OTC medicine. It's approved for reducing fever in people of all ages and used to relief pains associated with many parts of the body such as headache, muscle aches, arthritis, backache, toothaches, colds, and fevers. It relieves pain in mild arthritis but has no effect on the underlying inflammation and swelling of the joint [1]. It has analgesic properties comparable to those of aspirin and it is better tolerated than aspirin in patients in who suffer from excessive gastric acid secretion or prolongation of bleeding time [2]. The structure of Paracetamol consists of a benzene ring core, substituted by one hydroxyl group and the nitrogen atom of an amide group in the para (1,4) pattern [3]. Paracetamol is available in tablet, capsule, liquid suspension, suppository, intravenous and intramuscular dosage forms. Tablet comprises a mixture of active substances and excipients, usually in powder form, pressed or compacted from a powder into a solid dose. The excipients can include diluents, binders or granulating agents, lubricants to ensure efficient tableting, disintegrates to promote tablet break-up in the digestive tract, sweeteners or flavors to enhance taste and pigments to make the tablets visually attractive. A polymer coating is often applied to make the tablet smoother and easier to swallow, to control the release rate of the active ingredient and to enhance the tablet's appearance. Paracetamol syrups are a thick, viscous liquid consisting primarily of a solution of sugar in water, containing a resendable number of dissolved sugars. The sugar is mainly used to preserve the finished product, mask unpleasant taste of the active ingredient and enhance the flavor. The concentration should be between 65 and 67% in weight [4]. A lower percentage of sugar makes the syrup an excellent nutriment for yeast and other microorganisms. Preservatives used usually in syrups are parabens and benzoates, and are widely used in combination with one another in liquid, emulsion, cream, and lotion formulations. The antimicrobial effectiveness of these chemicals is greater against Gram-positive bacteria as compared to Gram-negative bacteria. Parabens are odorless and do not cause product discoloration [5]. Pharmaceutical products have been routinely contaminated with several types of microorganisms' right during and after manufacture [6,7]. Microbial contamination refers to the presence of undesired microbes or their metabolites, which may be pathogenic or may cause spoilage or degradation in an environment [8].

The interest on microbial contamination of drugs increased in recent years; firstly because of need to minimize spoilage, secondly is great awareness that newer types of medicaments frequently used nowadays are liable to contamination and thirdly the fatal and unfortunate consequences of contaminated drugs on patients whose immune system is already compromised by underlying diseases or therapy [9]. The microbiological quality of non-sterile pharmaceutical dosage forms is dependent on the bioburden of the raw materials, both active drug and excipient and strategy at every stage of production [10]. Quality of microorganisms is a concern, since the present of objectionable microorganisms as listed in USP are *S. aureus, P. aeruginosa, E.coli,* and *Salmonella spp.* are known pathogens and they shouldn't present in the pharmaceutical preparations. Some organisms may not be pathogenic or opportunistic pathogens, but can adversely impact the quality of the products. A guideline was issued to decide whether a given microorganism should be classified objectionable or not. Several studies around the world reported the contamination of various dosage forms [11-14].

The aim of the current study was to investigate the occurrence of microorganisms contaminating tablets, syrups and suppositories of paracetamol according to USP Chapter 61 by the mean of screening, as well as to identify the detected microorganisms using the required tests and also test the strength of preservative system in the tested products according the antimicrobial effectiveness test of USP Chapter 51.

MATERIALS AND METHODS

Samples collections and coding:

A total of 60 samples of paracetamol (tablet, syrups and suppository) were purchased from different pharmacies in Amman, Jordan. The expiration dates were reported for each sample. These include; (A) 30 samples of 500 mg tablet, (B) 15 syrup bottles (250mg /ml) and (C) 15 suppository samples (250 mg/ml).

Sample Preparation:

Bioburden test carried out according to USP chapters 61 and 62 guidelines to determine the microbial load [15,16]. The USP provides recommendations for dissolving or suspending the product so that a homogeneous sample is achieved.

All samples prepared under septic techniques using sterile materials. 10 mL or 10 g of the paracetamol test sample dissolved in 90 ml of SDW to make 1:10 dilution. For fatty products (suppositories), 5 ml of tween 80 were added to dissolve sample with aid of water bath at 45^{o}C. 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. SDW was used as a negative control [6].

Culturing the samples:

A 0.1 ml of each sample was streaked on the surface of nutrient media, soya bean casein digest and Sabouraud dextrose agar in duplicate. After culturing of samples, nutrient agar and soya bean casein digest plates were incubated at 35 °C for three to five days. While Sabouraud dextrose agar plates were incubated at 25 °C for five to seven days. Colonies observed on the streaking lines indicated the presence of microorganisms. Bacterial cultures were subsequently tested using Gram stain, catalase, oxidase, indole, citrate, KOH, nitrate, TSI and other tests. Identification of fungi was based on colonial morphology and microscopy [6,14].

Identifications of contaminating microorganisms:

Bacterial isolates were identified according to O'Hara *et al.,* [17]. These include the Gram stain which give the reaction and morphology in additional to biochemical tests. Automated identification was done by using Remel RapID ™ system [18].

Anti-microbial Effectiveness Test:

Referral to the current USP <51>, the Antimicrobial Effectiveness Test (AET) demonstrates the effectiveness of the preservative system in a product. A product is inoculated with a controlled quantity of specific microorganisms. The test then compares the level of microorganisms found on the test sample over a period of 28 days. According to USP <51>, five indicator organisms are utilized for the purpose of challenging the preservative system in a product. Three of the five USP indicator organisms which are *Escherichia coli* (ATCC No. 8739), *Pseudomonas aeruginosa* (ATCC No. 9027), and *Staphylococcus aureus* (ATCC No. 6538) address the growth of bacteria. *Candida albicans* (ATCC No. 10231) is the representative yeast, while *Aspergillus niger* (ATCC No. 16404) is a mold represented (Fig. 1) [6,14].

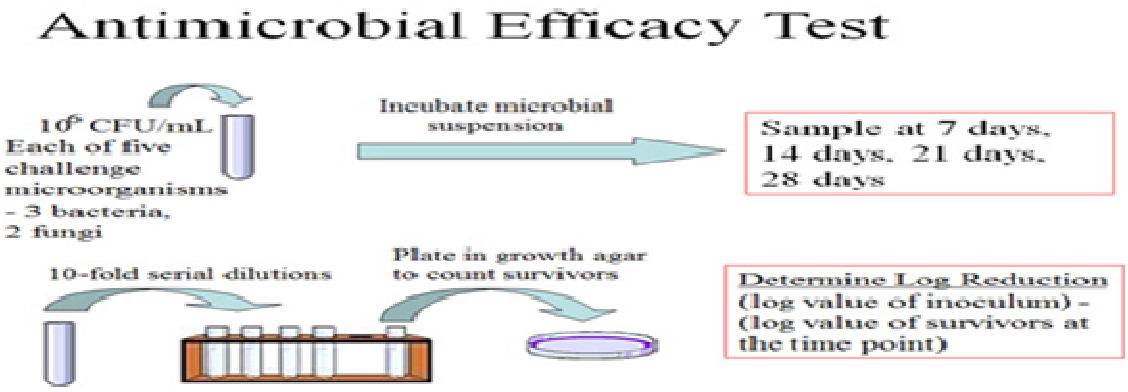

Figure (1): Summary of anti-microbial effectiveness test [14].

RESULTS

Isolation of objectionable and non-objectionable microorganisms: Table (1) illustrates the number of samples and the viable count of microorganisms present in theem. The highest bacterial count ($1x10^5$ cfu/g) was observed in tablet sample A25 and suppository sample C15.

Table (1): Microbial viable count of samples

Code	Sample size	No. of samples showed contamination	Range of Cfu/ml or g
Tablet(A)	30	9	50 cfu/g : 10^5 cfu/g
Syrup (B)	15	4	50 cfu/ml
Supp (C)	15	6	50 cfu/g : 10^5 cfu/g

Nineteen out of 60 samples tested possess varying degree of microbial contamination. Nine tablet samples namely A1, A4, A8, A12, A16, A22, A25, A26 and A30 were found to be contaminated with objectionable microorganisms like *S.aureus* and non-objectionable microorganisms like Gram positive *bacilli*. Some samples were contaminated with more than one type of microorganisms like A25 was contaminated with *S.aureus* and *B.brevis* , A4 contaminated with Gram negative *cocci* and *pencillium* while A26 contaminated with two types of fungi; *Racquet hyphae* and *pencillium*. Four syrup samples namely B4, B6, B13 and B14 contaminated by non-objectionable microorganisms like Gram positive bacilli and fungi. B13 was contaminated with *B.brevis* and *mucor* microbes. Suppositories samples namely C2, C7, C8, C13, C14 and C15 were contaminated with different types of microbes like *S.auricularis, S.epidermis, B.Subtilis, Enterobacteriace* and *Candida spp.* (Tables 2 and 3).

Table (2): Phenotypic and microscopic identification of coccus bacterial isolates

	Media	Colonial morphology	Microscopic appearance	Coccus type
A4	NA	Irregular, yellow flat colony, undulate margin	Mono and chains cocci	Gram negative cocci
A22	NA	circular, white raised colony, entire margin	Grape-like clusters	*S.epidermis*
A25	NA	circular, golden raised colony, entire margin	Grape-like clusters	*S.aureus*
B6	NA	circular, small orange colony, entire margin	Mono and tetra	*S. chromogenes*
C8	NA	circular, small white colony, entire margin	Tetra and clusters cocci	*S.auricularis*
C13	SCD	circular, white raised colony, entire margin	Grape-like clusters	*S.epidermis*

Table (3): Phenotypic and microscopic identification of bacillus bacterial isolates

	Media	Colonial morphology	Microscopic appearance	Bacillus type
A8	SCD	Circular, raised glistering buff colony entire margin	Chains and filaments	*B.circulans*
A12	NA	irregular, flat buff colony, undulate margin	Mono bacillus	*Corynebacterium*
A25	SCD	irregular, flat buff colony, undulate margin	Clusters of bacilli with Endospores	*B.brevis*
A26	NA	Circular, flat creamy colony, entire margin	Chains and filaments	*Enterobacteriace*
A30	NA	irregular, flat white colony, undulate margin	Clusters of bacilli with Endospore	*B.Subtilis*
B13	NA	Circular, flat yellow colony, undulate margin	Rods in chains or mono-appearance, endo-spores forming	*B.brevis*
B14	NA	Circular, flat yellow colony, undulate margin.	Chains of bacilli	*B.brevis*
C2	NA	Circular, flat buff colony undulate margin	Mostly mono bacilli and short chains	*B.Subtilis*
C2	SCD	Circular, flat buff colony undulate margin	Mostly mono bacilli and short chains	*B.Subtilis*
C14	NA	Circular, flat white colony, entire margin	Rods in chains appearance, endo-spores forming	*Enterobacteriace*

TYMC test applied for the three dosage forms resulted many fungal types. These are *pencillium, mucor, cephalosporum* and a *Racquet hyphae* (Table 4).

Table (4): Microscopic identification of fungi isolates

Sample	Media	Colonial morphology	Microscopic appearance	Fungus type
A4 A16 B4 C7	SCD SCD SDA SDA	Mature circular greenish colony with white margin sometimes	Single – celled spores in chains developing at the end of sterigma arising from metula of conidiphores which arise from mycelium	Penicillium
B13	NA	Circular white colony with black like hair, cottony and fuzzy	Brown globular sporangium arising from sporangiophore arising from mycelium. Spores are oval.	Mucor
A1	NA	Gray colony with white margin, cottony	Blue clusters of spores held together at the top of conidiophore arise from mycelium .	Cephalosporium
A16	SCD	Circular white colony , cottony and fuzzy	Black sporangium at the top of sporangiophore arising from mycelium . root like hyphae present and spores are brown.	*Racquet hyphae*

Table (5) summarizes all isolates resulted and illustrates the bacterial quantities and fungal counts for each product evaluated. Counts in general ranged between 50 to $1x10^5$ cfu/g or ml.

Table (5): Occurrence and count of microorganisms in the various samples

Microorganism isolated	Samples			Mean cfu/ml or g
	A	B	C	
S.aureus	A25	-	-	$1x10^5$ cfu/g
S.epidermis	A22	-	C13	75 cfu/g
S. chromogenes	-	B6	-	50 cfu/ml
S.auricularis	-	-	C8	50 cfu/g
-ve cocci	A4	-	-	50 cfu/g
B.circulans	A8	-	-	50 cfu/g
Corynebacterium	A12	-	-	50 cfu/g
B.brevis	A25	B13,B14	-	83 cfu/g or ml
B.Subtilis	A30	-	C2	75 cfu/g
Enterobacteriace	A26	-	C14	50 cfu/g
Cr. neoformans	-	-	C15	10^5 cfu/g
Pencillium	A4,A16	B4	C7	50 cfu/g or ml
Mucor	-	B13	-	50 cfu/g
Racquet hyphae	A16	-	-	50 cfu/g
Cephalosporum	A1	-	-	50 cfu/g

Anti-microbial Effectiveness Test (AET):

On day 14, samples that showed contamination on the screening study and then inoculated with microorganisms in this test show many visual changes around 80%; mainly color changing ranged from light brown to dark brown, this appear strongly in sample A25, also some tubes show fungal growth on the surface. On day 28, visual changing increases. Most of tubes color changed and some of them become very dark brown and fungal growth on surfaces increase. Some tubes show turbidity and formation of sedimentation. While samples that didn't show any contamination in screening study, it show very little visual changing, around 10% only include color changing. These observations are for tablet samples. For syrup samples, the contaminated samples become more liquefied and lose a degree of the viscosity, while samples that didn't show contamination have no changes and viscosity stay as it. For suppository samples, turbidity is the most observed changing.

Samples were tested for the viable count at the day 14's and day 28's according to the criteria of the AET. Table (6) shows all log value in the test points. The log reduction calculated as following:

Essential Log value (10^6) - Log value at the point of the test.

Table (6): Log values changing of the anti-microbial effectiveness test

	Log reduction at day 14's	Log reduction at day 28's
A28	Bacteria: 3 log reductions Yeast: 2 log reductions Fungi: 1 log reduction	Bacteria: no change from day 14's Yeast: no change from day 14's Fungi: log value increased 1 from day14's
A25	Bacteria: 2 log reductions Yeast: 2 log reductions Fungi: 1 log reduction	Bacteria: no change from day 14's Yeast: log value increased 1 from day14's Fungi: log value increased 1 from day14's
B7	Bacteria: 2 log reductions Yeast: 3 log reductions Fungi: 2 log reductions	Bacteria: no change from day 14's Yeast: no change from day 14's Fungi: no change from day 14's
B13	Bacteria: no reduction from 10^8 Yeast: 1 log reduction Fungi: 2 log reductions	Bacteria: no reduction from 10^8 Yeast: 2 log reductions Fungi: log value increased 1 from day14's
C6	Bacteria: 2 log reductions Yeast: 1 log reduction Fungi: no reduction from 10^8	Bacteria: no change from day 14's Yeast: log value reduced 1 from day14's Fungi: no reduction from 10^8
C15	Bacteria: 2 log reductions Yeast: no reduction from 10^8 Fungi: no reduction from 10^8	Bacteria: no change from day 14's Yeast: 1 log reduction Fungi: log value increased 1 from day14's

Samples which pass the screening test showed 2 to 3 log reduction in the microbial count, while samples that showed degree of microbial contamination showed only 1 log reduction and sometimes no reduction at all. In tablet and suppository samples, fungal count exceeded the initial count with 1 log. This appears also visually by fungal growth on the surfaces of test tubes.

DISCUSSION

In this study objectionable microorganisms were isolated from paracetamol dosage form, which causes rejection of product according to US pharmacopeia. The aerobic organisms were mainly Gram-positive Bacillus spp. The occurrence of Enterobacteriaceae and Staphylococci Spp. within tablet and suppository were observed, also Some Gram-positive spore-form bacilli were isolated from tablet samples. Among the fungi encountered with samples tested were *pencillium, mucor, cephalosporum* and *Racquet hyphae*. The aerobic mesophilic bacteria and fungi of most samples were however within the standard numerical limits for non-sterile oral preparations [6], while some products over the acceptable limits and should be rejected according to criteria mentioned by USP chapters 61 and 62. The total microbial count obtained from examined samples in this study ranged from $5x10^1$ to $1x10^5$ cfu/g or ml. Highest microbial count ($1x10^5$ cfu/g) reported in the current study were reported for *S.aureus* and *Candida spp*. While other majority of contaminants ranged between $5x10^1$ to $8.3x10^1$. Similar finding was reported by Kundol et al. [20].

The microbiological quality of nonsterile solid dosage forms is dependent on the bioburden of the

raw materials, both in the active ingredients and excipients [5,7,8,10-14].

S.epidermis it is part of human skin flora, and consequently part of human flora. It can also be found in the mucous membranes and its association with the drug could be due to talking, laughing, yawning and sneezing [4,9,17,20]. *S.aureus* is also normal skin flora on the skin and nasal passages. It is estimated that 20% of the human population are long-term carriers of *S.aureus* , which is the most common species of staphylococci to cause *Staph* infections [20]. However, *S. aureus* and *S. epidermidis* found as contaminants reflect easy contamination of processing unit. The organisms being normal floral of the body easily contaminate products during handling and processing by personnel [5,9].

Bacillus sp. is a normal flora of the soil so its presence within the drug tablets was most probably due to contamination during loading of the drug cartons, transportation, and off-loading and storage. Accumulation of used drug cartons in medicine stores as well as dirty surroundings could also offer free traffic flow of Bacillus into the drug tablets especially as this bacterium is a spore former. *Bacillus* sp. is ubiquitous and considered harmless because of their spoilage potential. Their presence in product suggests poor environmental hygiene during processing or contaminated raw materials [5,17].

The presence of fungi is mainly due to humidity of the tablets which is high than it should be. *Penicillium* is extremely common building materials, walls and wallpaper, floor, carpet and furniture dust. The spores of this mold are produced in dry chains and are exposed to the air hence their ease to become airborne [19]. The presence of *Penicillium* sp. in the tablets is a sign of spoilage. The hundreds of thousands of spores produced by this fungus are greatly encouraged by the relative humidity of the tablets which is high than it should be. Some of these tablets contain the requisite organic micro-elements which support the growth of the fungal isolates.

Contamination of products may affect their stability causing product degradation prior to expiration date and also it may lead to infections in children with weak immunology system [5]. Some of the raw materials utilized for the development of pharmaceutical formulations are based upon natural products that contain a high microbial load. The production process for these raw materials does not eliminate all microorganisms, therefore they are not sterile. Testing must be performed to determine the quality of these materials. The absence of objectionable microorganisms; *S. aureus, P. aeruginosa, E.coli,* and *Salmonella spp.* is required before raw materials can be used in pharmaceutical products. However some of the manufacturing process are designed to scientifically reduce the number of the microorganisms.

Different types of bacteria commonly found in pharmaceutical raw materials are *Lactobacillus spp., Pseudomonas spp., Bacillus spp., Streptococcus spp., Clostridium spp., E.coli, Agrobacterium spp.* etc. and mold such as *Cladosporium spp*. and *Fusarium spp.*

The three major pharmacopeias U.S. (USP) , European (EP) and Japanese (JP) have divided microbial limit testing into two different test; the quantitative test and the qualitative test. The quantitative test ascertains the number of microorganisms; bacteria, yeast and mold present in given pharmaceutical sample. The qualitative test determines the presence of specific pathogens indicators e.g *Salmonella spp., S.aureus, E.coli, P.aeurginosa,* and *Enterobacteriace* family which may cause disease to consumers or indicate the presence of other pathogenic bacteria. These indicators are representative microbial spp. of different types of bacteria populations [10-14]. For example *Salmonella spp.* and *E.coli* are Gram negative rod, capable of lactose fermentation, commonly found in fecal sources. The use of the four pathogen indicators doesn't mean that the presence of other microbes might not be a problem during quality evaluations as in case of our study, the presence of *Candida spp., Enterobacteriaceae* and fungal isolates which aren't mentioned as objectionable microorganisms but may cause a problem. However, as previously mentioned in introduction many factors will determine if there is a risk involved when these microbes are present. The great majority of microbial contamination for non-sterile products has been reported to be due to the presence of microbes in the raw materials or water or from poor practices during products manufacturing [5,18].

The possible adverse effect on health and the spoilage potential of these contaminants highlighted the need to reduce the degree of contamination of such products by establishing official guidelines such as good manufacturing practice (GMP) and ensuring compliance through regular monitoring of non-sterile products [15,16].

Microbial contamination of raw materials used to manufacture dry formulations (e.g., tablets) is often reduced by drug manufacturing processes such as granule drying and tablet compaction [20]. The amount of bioburden reduction is directly dependent on the process temperature, chemical properties of the drug formulation, tablet compression pressure, and metabolic properties of the contaminating microbes. For example, bacterial spores are less

susceptible to the harsh conditions encountered during tablet processing and the survival of *Bacillus subtilis* spores found in raw materials has been studied and documented.

Personnel's who handle drugs especially exposed tablets must routinely put on properly laundered over-all or laboratory coats, hand gloves and nose masks to exclude excessive droplets from nasal passages and buccal cavity that are generally associated with sneezing, coughing or talking. Hand washing facilities must be available and usable. The toilets must be cleaned regularly and adequately disinfected. In addition, persons employed to clean the toilets and bathrooms should be restricted from handling drug tablets. Air conditioners should be installed in all drug dispensing stores as this will help reduce the growth of microorganism. Above all, the use more cheaply designed multi-dose packs which dispense tablets individually through a shutter release aperture without having to open the container cover would ultimately revolutionize large package dispensing drug tablets.

REFERENCES

1. Benista MJB. and Nowak JZ. (2014). Paracetamol: mechanism of action, applications and safety concern. *Acta. Poloniae. Pharmaceutica ñ Drug. Res.* 71(1):11-23.

2. Hinz B.; Cheremina O. and Brune K. (2008). Acetaminophen (paracetamol) is a selective cyclooxygenase-2 inhibitor in man. *The FASEB Journal: official publication of the Federation of American Societies for Experimental Biology* 22 (2): 383–390.

3. Mwambete KD. (2011). Incidence of fungal contamination of tablets available in Dar Es Salaam market-Tanzania. *J. Pharmacy Res.*4(3):868-870.

4. Hapsari I.; Marchaban M.; Wiedyaningsih C. and Kristina SA. (2018). The Extemporaneous Compounding at Primary Health Care Centers: Characteristic and Personnel. *Glob. J. Health. Sci.* 10(9):112.

5. Al-Aswad RS. and Al-Shammari AJN (2017). Efficacy of antimicrobial preservatives in pharmaceutical dosage forms during shelf life. Int. J. Sci. Technol. 12(3): 11-19.

6. Clontz L. (2009). Microbial Limit and Bioburden. Tests Validation Approaches and Global Requirements. 2nd ed. New York, London: Taylor & Francis Group; pp.:66-78.

7. Mugoyela V. and Mwambete KD. (2010). Microbial contamination of non-sterile pharmaceuticals in the public hospital settings. *Ther. Clin. Risk. Manag.* 6: 443-448.

8. Baird RM. (2004). Microbial spoilage, infection risk and contamination control. In: Denyer SP, Hodges NA, Gorman SP, Hugo W, and Russell's A (eds), Pharmaceutical Microbiology. 17th ed. Blackwell Scientific Publications: London, U.K, pp. 262-284.

9. U.S. Food and Drug Administration (FDA). (2017). FDA's Human Drug Compounding Progress Report: Three Years After Enactment of the Drug Quality and Security Act. Available From: https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/PharmacyCompounding/UCM536 549.pdf

10. Eissa ME. (2016). Distribution of bacterial contamination in non-sterile pharmaceutical materials and assessment of its risk to the health of the final consumers quantitatively. *J. Basic Appl. Sci.* 5(3):217-230.

11. El-Housseiny R.; Aboulwafa MM.; Aboulwafa EW. and Hassouna N. (2013). Recovery and detection of microbial contaminants in some non-sterile pharmaceutical products. *J. Clin. Microbiol*. 4: 736-742.

12. Gad GF.; Aly RA. and Ashour MSE. (2011). Microbial evaluation of some non-sterile pharmaceutical preparations commonly used in the Egyptian market. *Trop. J. Pharm. Res.* 10: 437-445.

13. Khanom S.; Das KK.; Banik S. and Noor R. (2013). Microbiological analysis of liquid oral drugs available in Bangladesh. *Int. J. Pharm. Sci.* 5:479-482

14. Al-Jaberi GF. and Al-Shammari AJN. (2016). Contamination of baby creams and lotions with objectionable and non-objectionable microorganisms in the Jordanian market. *Int. J. Sci. Technol.* 11(4):52-57.

15. USP 36-NF 31, Chapter (61). (2014). Microbial examination of non-sterile products: Microbial enumeration test. United States Pharmacopeial 36/ National formulary 31, Baltimore, MD, USA.

16. USP 36-NF 31, Chapter (51). (2014). Antimicrobial Effectiveness Testing. United States Pharmacopeial 36/ National formulary 31, Baltimore, MD, USA.

17. Hapsari I.; Hadimartona M.; Wiedyaningsih C. and Kristina SA. (2019). Microbial contamination on dosage form of non-sterile semi-solid extemporaneous compounding in primary health care centers. *Int. Med. J.* 24(4): 317-324.

18. O'Hara CM.; Weinstein MP. and Miller J M. (2003). Manual and automated systems for detection and identification of microorganisms, p. 185-207. *In* P. R. Murray, E. J. Baron, M. A. Pfaller, J. H. Jorgensen, and R. H. Yolken (ed.), Manual of clinical microbiology, 8th ed. American Society for Microbiology, Washington, D.C.

19. Al-Mosely ML. and Al-Shammari AJN. (2018). Determination of microbial limits for pharmaceutical dosage forms by chromogenic agar. *Int. J. Sci. Technol.* 13(2):4-14.
20. Kundol NK.; Sultan C.; Islam MS.; Ali A. and Khatuna M. *et al*.(2018). Microbial and physicochemical evaluations of paracetamol in different brands of analgesic syrups sold in Bangladesh. *J.Innov.Pharmact. Biolo. Sci. (JIPBS).* 5(1):46-50.

Detection of Enrofloxacin Residue in Chicken Tissues

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ABSTRACT

The use of enrofloxacin in chicken may leave drug residues in food consumed by peoples. These residues represent a risk to public health. In this study, two simple and fast methods were applied for detection of antimicrobial residue (Enrofloxacin was selected for the study) in chicken meat tissues (liver tissue, thigh tissue, and breast tissue). These methods were: the Thin Layer Chromatography (TLC) according to its sensitivity and accuracy for monitoring low amounts of different biological and chemicals. Illumination of antibiotics against UV light helps as a simple detector for this mean. The second method was microbiological assay for detection of the residues. A total of 75 samples poultry products; liver, breast and thigh muscle samples, were tested for the presence of enrofloxacin residue. Out of 75 chicken samples, 18(24%) were positive for enrofloxacin. Liver and breast muscle samples had the highest percentage of antibiotic detected (28%), followed by for thigh muscle (16%). The tested antibiotic enrofloxacin of the tested group (quinolone) was able to induce an inhibition zone. The mean diameters of inhibition zones of the majority of tested antibiotic were ≥ 36 mm with coefficient of variation (CV) of 3 %, based on the measured of MIQ and MRL of tested antibiotic. Seven standard solutions, of concentrations varying from 625 to 3.500 ng/ml, were used for the evaluation of the MIQ. The concentration of quinolones creates the larger inhibition zone. Concretely, Enrofloxacin produced an inhibition zone higher than 2 mm at concentrations between 37.50 and 50 ng/disk. Extracted samples taken from chicken samples were applied to TLC. Out of 75 samples used, only 15 (20%) were shows detectable bands after exposed to UV light. Seven liver samples, six breast muscle and 2 thigh muscles were positive. We concluded that enrofloxacin residue was found in slaughter chicken consumed by people in Jordan and the two methods used for detection were applicable.

Keywords: Enrofloxacin, chicken tissues, TLC, Microbiological assays

INTRODUCTION

Shortly after antibiotics were developed, they were used in veterinary medicine at first time to treat mastitis in dairy cows [1-3]. Moreover, during 1940s, the growth promoter effect of antibiotics was discovered when it was observed that animals fed dried mycelia of *Streptomyces aureofaciens* containing chlortetracycline residues improved their growth [4]. The mechanism of action of antibiotics as growth promoters is related to interactions with intestinal microbial population [5].

Actually, antibiotics are used either to control any infectious disease or to treat sick animals, as a part of integrated disease management approach that incur porates other management processes such as minimizing the external sources of infection among animals at farms and other biosecurity measures [6,7]. In poultry, the uses of antibiotics had facilitated their efficient production, as well as enhanced the health and wellbeing of poultry by reducing the incidence of diseases [8].

Out of the most common widely used antibiotics in poultry is Enrofloxacin (ENR) (Baytril- Bayer company- Germany), a fluoroquinolone with a broad antibacterial spectrum and high bactericidal activity against major pathogenic bacteria found in sick animals [9]. The widespread administration of these drugs in veterinary medicine represents a potential risk, because their residues may persist in edible animal tissues and may result in the development of drug-resistant bacterial strains or allergies [8, 10,11].

Human as a non-target organism of these drugs receives different amounts of them as residue, which can cause private changes in the intestine microflora and elimination of some useful bacterial strains. Another danger of receiving antibiotic residues is the possible occurrence of microbial resistance of body microflora to common antibiotics which may cause serious problems if human suffers any of the microbial infections [1,5-7,11]. Therefore, the determination of antibiotic residues in chicken and other animal products used for human consumption is an important task. Several methods for detection and quantification of ENR and CPX in biological fluids and tissues have been proposed, such as an immunosorbent assay, voltammetry, spectrophotometry, capillary electrophoresis, and liquid chromatographic methods [12-18]. It is necessary to implement simply and reliable screening systems for the detection of antibiotics as tools in assuring the safety of food products.

However, most of these methods involve a preliminary extraction step followed by a second clean-up step with liquid-liquid extraction or solid-phase extraction (SPE), which make these approaches too complicated, time-consuming, and use large amounts of organic solvents [17].

The current study had focused into two methods used for detection of antimicrobial residues (Enrofloxacin) in chicken meat tissues; microbiological assay and TLC methods with selected samples of fresh chicken meat from Jordanian markets.

MATERIALS AND METHODS

Sample Collection:

Seventy-five samples of fresh poultry products (25 livers, 25 thigh muscles, 25 breast muscle) were selected from different markets and slaughter houses in Amman city, Jordan.

TLC:

TLC Silica plates with 0.25 mm. thickness (Merck, Germany) were activated in 120°C for two hours before use [19,20].

Standard preparation:

Raw antibacterial Enrofloxacin was prepared by dissolving of 0.1 gm of each powder in 4 ml methanol [21].

Antibiotics extraction for TLC analysis:

Ten grams of each sample of fresh poultry products were mixed with 10 ml ethanol (GCC Laboratory reagents), crashed and squeezed fine in a Chinese mortar. The solution transferred to 15 ml falcon centrifuge tubes and centrifuged at 7000 rpm for 10 minutes. The clear supernatant transferred to fresh glass test tubes and evaporated in rotary evaporator. The drying deposits dissolved in 0.2 ml methanol (GCC Laboratory reagents). The samples were pointedly on silica plates [19,21]. About 50 µl of methanol dissolved deposits were pointed on silica plates. Treated plates transferred to TLC tank containing acetone-methanol (1:1) as mobile phase.

After reaching of solution front to end of plates, chromatograms dried and observed on UV light at 256 nm [21].

Microbial inhibition assay:

Antibiotic standard used in this study was provided by Sigma-Aldrich (St Louis, MO, USA). Stock solutions (1mg/ml) was prepared by dissolving quinolones in NH4OH 2M before methanol addition. Standard working solutions: Standard working solutions were prepared by diluting stock solutions in purified sterile water.

Culture media:
Culture media used were Standard II Nutrient Agar for microbiology (Merck 1.07883, Darmstadt, Germany). Media were prepared as recommended by [15,22] with two modifications (0,6% dextrose and 0.4 µg TMP/ml culture). *Escherichia coli* used as an indicator strain *(*ATCC No. 8739). Three blank samples were taken from house chicken (local breed), not exposed to any antibiotic treatment.

Sample extraction for microbiological assay:
The extraction method was adapted from the premi-test as described by Stead *et al,* [23]. Three grams of each type of chicken tissues were extracted in Acetonitrile/ Acetone (70:30 V/V) under rotative shaking during 10 minutes. The mixture was then centrifuged at 300 rpm for 10 minutes at 15C. The supernatant was transferred into a clean conical tube and evaporated to dryness.

Microbiological test:
The extract was centrifuged again and the 50 µl of the supernatant was applied on paper disc on the seeded agar plates with *E.coli*. Plates were then incubated for 24 h at 30°C, before to measure inhibitions zones. Positive or suspect results were reported, if the diameter of the inhibition zone (including the paper disc) was equal or higher than 16 mm, or if the size of the inhibition zone around the paper disc was equal or higher than 2 mm [24].

Method optimization:
Standard quality control were performed with 50 µl of each of the 7 standard solutions at a concentration of 20 µg/ml (=1 µg of antibiotic per disc). Then MIQ (Minimum Inhibitory Quantity) is the minimum quantity of antibiotic able to produce an inhibition zone which is equal or bigger than 2 mm. The MIQ was determined by using 7 standard solutions in the range of concentration 20 µg/ml. On the basis of the MIQ and the MRL (maximum residue limit) of each antibiotic, the minimal chicken tissue quantity (MCQ) to be used for the analysis was determined using the following formula:

MCQ (g) = MIQ (ng) x MRL-1 (g/ng)

Then LODs (Limit of detection) for each antibiotic was determined by analyzing 20 spiked samples. The method was validated following the "Guide for analytical validation of screening methods" [25]. The accuracy, the sensitivity and the selectivity were determined by analyzing 20 "blank" samples and 20 spiked samples.

Statistical analysis:
SPSS and calculation of mean and standard deviations, as well as frequency and percentage were applied throughout this study.

RESULTS

Detection of enrofloxacin residue in chicken tissue:
Out of 75 chicken samples taken from slaughtered chicken, 18 were positive. The test performed for enrofloxacin and for each type of fresh poultry product, liver, breast and thigh muscle samples are shown in table (1). Liver and breast muscle samples had the highest percentage of antibiotic detected (28%), followed by for thigh muscle (16%).

Table (1): Percentage of positive (%) samples of enrofloxacin

Tissue type	Number of samples	No. of positive	Percentage of positive
Breast muscle	25	7	28%
Thigh muscle	25	4	16%
liver	25	7	28%
Total	75	18	24%

Standard Quality Controls
To evaluate the plate quality and the sensitivity of *E.coli* to tested antibiotic, 50 µl of the standard solution at the concentration of 20 µg/ml were analyzed on 12 mm diameter paper discs [26]. The tested antibiotic was tested with 12 repetitions (3 repetitions in 4 independent series of disk preparation) on 4 different days (4 independent preparations of medium). The tested antibiotic enrofloxacin of the tested group (quinolone) was able to induce an inhibition zone. The mean diameters of inhibition zones of the majority of tested antibiotic were ≥ 36 mm with coefficient of variation (CV) of 3 % (Table 2).

Table (2): Diameters of inhibition zones generated by antibiotic standard (1 µg op standard/disc)

Group	Antibiotic	Diameters of inhibition zones (n=12) (mm)			
Quinolones	Enrofloxacin	Xa+/-sa	Max	Min	CV (%)
		36+/-1	38	34	3

Xa , sa = mean diameter of inhibition zone and standard deviation for enrofloxacin
Test evaluation with standard antibiotic solutions

Determination of the minimal inhibitory quantity (MIQ):

The MIQ is very necessary for establishing and adjusting the sample extraction procedure. Based on measured MIQ and MRL of the tested antibiotic (fixed by regulation 2377/90/CEE).

Seven standard solutions, of concentrations varying from 625 to 3.500 ng/ml, were used for the evaluation of the MIQ. Fifty µl of standard solution was dripped on the paper disks laid on the Petri plate. Each standard solution at each concentration was tested in 8 repetitions with 4 series of Petri plate, on 4 different days. As expected, the width of the inhibition zones around the disks loaded with 50 µl of standard solution varied in function of concentrations. The dose-response curves of the tested antibiotic group were different. At the same concentration, quinolones could create the larger inhibition zone. Concretely, Enrofloxacin produced an inhibition zone higher than 2 mm at concentrations between 37.50 and 50 ng/disk. Thus, the indicator *E.coli* strain was the most sensitive to Enrofloxacin (MIQ varied from 37.5 to 50 ng/disk).

Determination of the minimal quantity for an extraction:

In order to detect antibiotic residue in samples, an extraction procedure is applied. Based on the extraction procedure, the maximum residue limit (MRL) fixed by EU and the MIQ (ng in 50 µl/disk). The minimal chicken quantity (MSQ) was calculated to be taken for an extraction using the formula mentioned in the material and methods section (Table 3).

Table (3): Determining minimal chicken quantity to be sampled for an extraction

Enrofloxacin	**MRLs (*)** (ng/g)	**MIQ** (ng/disk)	Minimal chicken quantity to be sampled for extraction (In 50µl of the final extraction solution) (g)	Minimal shrimp quantity to be sampled for an extraction (***) (g)
	100	37,5 - 50,0	0,5000	1,5 - 2,0
*(*): MRLs fixed by regulation 2377/90/CEE (CE, 1990) **: without fixed MRL* *(***): if dry residue after the last evaporation step is dissolved in 200 µl of methanol*				

If the dry residue after the evaporation step following the extraction is recovered in 200 µl of methanol, and if 50 µl are applied on a disk, the minimal chicken quantity for an extraction will be 4 times the MSQ.

Detection of Enrofloxacin residue in chicken tissue by TLC:

Extracted samples taken from chicken samples were applied to TLC. Out of 75 samples used, only 15 (20%) were shows detectable bands after exposed to UV light. Seven liver samples, six breast muscle and 2 thigh muscles were positive.

Detection of Enrofloxacin in different chicken corpses by thin layer chromatography showed clear bands after visualization by UV light, free samples taken from local breed that did not expose to any treatment by antibiotics, does not show any band. Standard enrofloxacin run on each plate and treated equally, shows one band run in the same level of tested samples. The similarities between Rf value of detected bands from suspected samples with standards led us to sure that there are near link and correlation between them. The intensity of bands extracted from liver was higher than others.

DISCUSSION

Enrofloxacin (ENR) is a fluoroquinolone with a broad antibacterial spectrum and high bactericidal activity against major pathogenic bacteria found in diseased animals. The pharmacokinetics of ENR is characterized by good absorption and extensive distribution into various animal fluids and tissues. In several animal species, including chicken, ENR is de-ethylated to its primary metabolite, ciprofloxacin (CPX), and both ENR and CPX are found in the muscle and tissue of animals receiving ENR [14,16,27]. The widespread administration of these drugs in veterinary medicine represents a potential risk, because their residues may persist in edible animal tissues and may result in the development of drug-resistant bacterial strains or allergies. Therefore, the determination of their residues in meat and other animal products used for human consumption is an important task. The federal government, to ensure the safety of the food supply, establishes antibiotic residue tolerances in edible animal tissues and determines the target tissues (e.g., muscle) for residue monitoring. However, when this study selected the muscle as the target tissue, because the federal government does not

specify which type of muscle tissue is used for monitoring (e.g., breast versus thigh) [14,18, 28,29]. If specific muscle tissues incorporate residues at higher concentrations, these tissues should be selected for residue monitoring. Results indicate, that enrofloxacin, not incorporated in all muscle tissues at the same concentrations. This finding was revealed by zone inhibition size as judged by mm. compared with those obtained by the same standard solution. These results may be helpful to regulatory agencies as they determine what tissues are to be monitored to ensure that the established residue safety tolerance levels are not exceeded. Both methods used in this study shows good detectable measurement. The calculations of MIQ and MRL clearly this situation as indicated in table (2). In fact, the quantity of the residual antibiotics is mainly concerned with the problem of bacterial resistance. The quantity above the maximum residue limit (MRL) of a specific antibiotic is important for causing the bacterial resistance [22].

Similar results were reported by Clanjak *et al*. [12], they found that both microbiological and ELISA method achieve equally positive results in the detection of the allowed quantities of enrofloxacin residues, although they are different measures (mm or ppb) of the same phenomenon.

Analytical Thin Layer Chromatography (TLC), which employed in the study was not tasked for measuring concentration of antibiotic residues, but for detection their presence in tested chicken products. This technique is simple, exact and non-expensive which can execute easily in most laboratories, but with less accuracy than HPLC. However, the current study showed that about third of the tested samples were positive (24%). In any case, these findings were differing to those obtained by others [15,30]. In Iran, Tajick and Shohreh [15] found that more than 50% of poultry meat samples had noticeable antibiotic residue. Higher percentage (77.5%), was reported by Jammoul and El Darra [31], through examining 80 chicken muscles in Lebanon. Higher percentage (96%) was recorded in Canada during 1991-1992 through examination 961 poultry samples. Nevertheless, most laboratories still give too little attention to the reliability of their screening methods. Of course, the validation characteristics differ between screening and confirmation methods [25]. The causes of the differences between detection limits of antibiotics in aqueous solution and in spiked muscle tissue were not investigated. With some antibiotics the difference was only observed at low levels. This can be due to variation in diffusion into the agar layer. The phenomenon was not observed with high levels of tetracyclines and the fluoroquinolones ciprofloxacin and enrofloxacin. It was seen, but to a lesser extent, with beta-lactam antibiotics and the quinolone flumequine.

The Four-plate test (FPT) is one of the microbiologic methods for detecting antibiotic residues in food stuff, which is based on inhibition zone formation around the sample in four culture media with different pH and test bacteria [14]. The method demonstrated that the FPT is not suited for detection of many antibiotics in muscle tissue. A false negative result can be defined as follows: a negative result from a sample spiked with a specified level of analyte, preferentially corresponding with the MRL or safe level. The number of false negative results was 100% for several antibiotics when meat was added to the standard compound, although the sensitivity of the plates was optimal. Sulfadimidine will not be detected with the FPT. The detection limit of sulfadimidine is approximately 50 ng; this corresponds with 0.5 mg kg21 tissue, which is far above the MRL.

Only tetracyclines and quinolones are detected nearly equally well with as without meat. Probably all samples with levels of oxytetracycline, doxycycline, tetracycline, chlortetracycline, enrofloxacin or ciprofloxacin equal to or higher than the MRL, will be detected. This applies only to spiked samples; it is still possible that residues of one or more antibiotics belonging to these groups do not diffuse completely into the medium, when they are present in naturally contaminated samples [14,15].

A possible explanation for the nearly total absence of inhibitory zones with high levels of sulfadimidine, ceftiofur and macrolides is a change in composition of the medium. The modified FPT prescribes the use of a thin layer of agar medium; only 5 ml are poured into 90 mm plates, which is just enough to cover the surface. Thin agar layers cause higher sensitivities for aqueous solutions of antibiotics [10]. On these thin layers 2 mm thick pieces of frozen meat are laid. Soluble cell contents as well as the antibiotics diffuse into the agar. Normal muscle tissue has a pH lower than 6. It is clear that the pH of the medium is influenced by the matrix. Furthermore, the diffusion of proteins or other nutrients into the medium may influence the detection of antibiotics too. The definition of matrix effect, as used in analytical chemistry, is an influence of the matrix on the sensitivity of the sensor [10]. Obviously, this is the case with the inhibition test: very often test strains grow more abundantly around blank meat disks, and this phenomenon was even observed around the spiked tissues [11,29].

The addition of a plate seeded with *E. coli* to the system will facilitate the detection of quinolones and fluoroquinolones. The use of *E. coli* as an indicator strain however has its shortcomings. It has difficulty detecting oxalinic acid and flumequine residues at sufficiently low levels, while especially flumequine is

commonly used in poultry. Enrofloxacin, on the other hand, can be detected extremely sensitive with respect to MRL values. Therefore, the use of *E. coli* for screening purposes will easily lead to false compliant results with respect to flumequine, but will also yield [23].

Many false non-compliant results when enrofloxacin is present in a sample. These statements are based upon the demonstration of an effect of the tissue matrix, added to the antibiotic standards. Further evidence is needed that the majority of naturally contaminated or incurred samples, with residue levels equal to the MRL will also reacting positive [28].

REFERENCES

1. Food and Agriculture Organization of the United Nations; World Health Organization. (2009). Evaluation of certain veterinary drug residues in food. *World Health Organ Tech Rep Ser.* 954: 1-134.

2. Chen J.; Ying GG. and Deng WJ. (2019). Antibiotic residues in food: Extraction, analysis, and human health concerns. *Agri. Food Chem.* 67(27): 7569-7586.

3. Patel T.; Marmulak T.; Gehring R.; Pitesky M.; Clapham MO. and Tell LA. (2018). Drug residues in poultry meat: A literature review of commonly used veterinary antibacterial and anthelmintics used in poultry. *Vet. Pharmacol. Ther.* 41(6): 761-789.

4. Dibner JJ. and Richards JD. (2005). Antibiotic growth promoters in agriculture: History and mode of action. *Poult. Sci.* 84:634–643.

5. Witte W. (1998). Medical consequences of antibiotic use in agriculture. *Sci.* 279: 996-997.

6. Bacanli M. and Basaran N. (2019). Importance of antibiotic residues in animal food. *Food Chem Toxicol.* 124: 462-466

7. Allen HK. and Stanton TB. (2014). Altered egos: antibiotic effects on food animal microbiomes. *Annu. Rev. Microbiol.* 68:297–315.

8. Agyare C.; Boamah VE. and Zumbi CN. (2018). Antibiotic Use in Poultry Production and its Effects on Bacterial Resistance. Intech Open. Chapter 3. http://dx.doi.org/10.5772/intechopen.79371

9. Al-Shammari AJN. (1995). Comparison between timulin, tylosin and Enrofloxacin in treatment of chicken experimental infected by *M. gallisepticum. Veterinar.* 5(1): 18-22.

10. Khannazer H. and Kahba H. (1999). Investigation of antibiotic and sulfonamide residues in poultry with FPT and thermal effects on its. *J. Res. Develop*. 43:62-65.

11. Jayalakshmi K.; Paramasivam M.; Sasikala M.; Tamilam TV. and Sumithra A. (2017). Review on antibiotic residues in animal products and its impact on environments and human health. *J. Entomol. Zool. Stu.* 5 (3): 27.

12. Clanjak E.; Smajlovic M.; Faruk C. et al. (2011). Detection of enrofloxacin residues in chicken meat by microbiological (growth inhibition test) and ELISA method after experimental prophylactic and therapeutic application. Animal Protection and Welfare. *The 18th Sci.conf. Int. Parti.* 20-21. Czech Republic.

13. Berna K. and Sukran C. (2008). Screening for antibiotic residues in the trout by the Four Plate test. Premi test and ElISA test. *Euro. Food. Res. Technol.* 226:795-799.

14. Pena A.; Silva LJG.; Pereira A.; Meisel L. and Lino CM.(2010). Determination of fluoroquinolone residues in poultry muscle in Portugal. *Anal. Bioanal. Chem.* 397: 2615–2621.

15. Sajid A.; Kashif N.; Kifayat N. and Ahmad S. (2016). Detection of antibiotic residues in poultry meat. *Pak .J. Pharm. Sci.* 29(5): 1691-1694.

16. Kotretsu SI. (2004). Determination of aminoglycosides and quinolones in food using tandem mass spectrometry: A review. *Crit. Rev. Food Sci. Nutr.* 44: 173-184.

17. Tajick MA. and Shohreh B. (2006). Detection of Antibiotics Residue in Chicken Meat Using TLC. *Int. J Poult. Sci.* 5(7):611-612.

18. Ramatla T.; Ngoma L.; Adetunji M. and Mwanza M. (2017). Evaluation of Antibiotic Residues in Raw Meat Using Different Analytical Methods. *Antibiotics.* 6,34.

19. Homans AL. and Fuchs A. (1970). Direct bioautography on thin-layer chromatograms as a method for detecting fungitoxic substances. J. *Chromatogr.* 51:327-329.

20. Fried B. and Sherman J. (1982). Thin-Layer Chromatography: Techniques and Applications. Marcel Dekker, New York.

21. Thangadu S.; Shukla SK. and Anjaneyulu Y. (2002). Separation and detection of certain B- lactams and fluroquinolone antibiotic drugs by thin layer chromatography. *Analyt. Sci.* 18: 97-100.

22. Pikkemaat MG.; Sabrina O.; Jan S.; Michel R. and Harry JV. (2008). A new microbial screening method for the detection of antimicrobial residues in slaughter animals: The Nouws antibiotic test (NAT screening). *Food Control.* 19(8): 781-789.

23. Stead S.; Sharman M.; Tarbin JA.; Gibson E.; Richmond S.; Stark J. and Geijp E. (2004). Meeting maximum residue limits: an improved screening technique for the rapid detection of antimicrobial residues in animal food products. *Food Addit. Contaminant.* 21(3):216-221.

24. Biemer JJ. (1973). Antimicrobial susceptibility testing by the Kirby-Bauer disc diffusion method. *Ann. Clin. Lab. Sci.* 3(2):135-140.

25. Association of Official Analytical Chemists (AOAC). (2002). Guidelines for single laboratory validation of chemical methods for dietary supplements and botanicals. AOAC International: Maryland, USA. P. 22.
26. Salehi TZ. and Bonab SF. (2006). Antibiotic susceptibility pattern of E. coli strains isolated from chickens with coli-septicemia. *Int. J. Poultr. Sci*. 5(7): 677-684.
27. Noppe CV. and De Zutter L. (2007). Microbiological detection of 10 quinolone antibiotic residues and its application to artificially contaminated poultry samples. *Food Addit. Contaminant.* 24(3): 252-257
28. Gundogan N.; Citak S.; Yucel N. and Devren A. (2005). A note on the incidence and antibiotic resistance of Staphylococcus aureus isolated from meat and chicken samples. *Meat. Sci.* 69:807–810.
29. Cheong CK.; Hajeb P.; Jinap S. and Ismail-Fitry MR. (2010). Sulfonamides determination in chicken meat products from Malaysia. *Int. Food Res. J.* 17:885–892.
30. Muaz K.; Riaz M.; Akhtar S.; Park S. and Ismail A. (2018). Antibiotic Residues in Chicken Meat: Global Prevalence, Threats, and Decontamination Strategies: A Review. *J. Food Prot.* 81:619–627.
31. Jammoul A. and El-Darra N. (2019). Evaluation of Antibiotics Residues in Chicken Meat Samples in Lebanon. *Antibiotics.* 8(69):1-11.

Determination of thermodynamic parameters and studying the stability of some aromatic complexes derived from 4-dimethyl aminobenzaldehyde with dinitro aniline reagent

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ABSTRACT

This study was built basically on the preparation of aromatic azoimine complexes resulting from the coupling reaction of Schiff bases with diazotized dinitroaniline reagent. Ultraviolet spectra, infrared spectra, and melting points are among the most important physical methods used to identify the right compounds prepared in this study. The stoichiometric ratios of the components of the complex were determined using the molar ratio method, the ratio obtained was (1:1) for all the studied complexes. Finally, the factors that affect the stability constant values were studied, namely;

Effect of acidity function, the stability constant for each prepared complexes were determined at acidic function (pH) and various temperatures. The value of the stability constants indicates the stable Azo complex preparations. The stability constant was calculated at the range of temperature (313–273oK) which facilitate to know that the reactions of formation of azo dyes was spontaneous and exothermic from the negative values of (ΔG and ΔH) respectively, as well as the negative (ΔS) value that supports what was mentioned before.

Effect of Structure:

The location and the type of function group also has an effect on the values of the stability constants of the prepared complexes, and this was proved by the variation in the values of the stability constants.

Keywords: Stability Constant, Stoichiometry Azo Imine Complex Spectrophotometry.

INTRODUCTION

The imine is known as an organic chemical compound that includes a double bond linking carbon with nitrogen (-C=N-), and it replaces an oxygen atom with a nitrogen atom to turn ketones and aldehydes into organic compounds, which are imines [1].

Schiff bases are condensation products of aliphatic or aromatic primary amines with carbonyl compounds such as aldehydes or ketones. Schiff bases contain a common structural-functional group for these compounds, the azomethine group, whose general formula is RHC=N-R1, where R and R1 are alkyls, aryl, cycloalkyl groups, or heterocyclic groups, which can be substituted by different substituents. There is competition between azomethane functional groups C=N and carbonyl group C=O, where carbonyl excels in the ability to form complexes with positive ions [2]. These compounds are also known as azomethines. The presence of the ion pair of electrons in the sp2 hybrid orbital of the nitrogen atom of the azomethine group is important in terms of its effect on the basic properties of the compounds containing a group of azomethane [3,4]. The stability and properties of Schiff bases are greatly influenced by the carbonyl compounds and the type of amine derived from them, whether aromatic or aliphatic. The presence of functional groups that have large electrical negativity related to the imine nitrogen reduces the polarization formed by electrostatic repulsion and the reason is due to the juxtaposition of negative similar charges [5].

In a recent study, stable complexes were prepared from coupling Schiff bases with transition metal ions such as Cu2+, Pd2+, U(VI), etc... It was concluded that these complexes are usually stable at heating and many researchers were able to describe their thermal decomposition [6].

There are several modern methods for producing Schiff bases, including environmentally friendly ones, as in the green production method. It is economically feasible, without pollutants, easy, and its yield in terms of production is high. The reaction medium is water. The preparation is done from the reaction of 1,2-diamino benzene with aromatic dehydrate, and we get the product by filtering and then washing and drying [7]. Choosing the appropriate solvent and the optimum reaction conditions obtained have the great effect of producing Schiff bases in this way with excellent yields and one step. Also, one of the advantages of this method is to reduce the reaction time, which means saving the energy needed for production, as well as increasing the conversion rate and reducing waste. The output was confirmed by IR, Mass spectra, and 1HNMR data [8]. In recent years, interest in imines has focused on the health field, as the number of people suffering from cancer and multidrug-resistant infections has increased sharply, leaving humanity with no choice but to search for new treatment options and strategies. Although cancer is considered the leading cause of death worldwide, Schiff bases have been a topic of great interest, due to their versatile chelating properties, inherent biological activities, and flexibility in modifying the structure to tune it for a particular biological application. Research in Schiff base-based metal complexes is underway to develop new anticancer and antimicrobial chemotherapies and because the anticancer and antimicrobial targets are different, structurally heterogeneous Schiff bases can be modified to achieve the wanted molecule, which targets a specific disease [9]. The diazotization reaction is taking place to form azo complexes in optimal conditions, the most important of which is the temperature, which should be close to (zero Celsius), and this degree gives stability to the resulting salts also increases the solubility of nitrous acid and releases it as a gas [10]. The electron-pushing groups in the amine increase the stability of the diazonium salt, and on the contrary, the electron-withdrawing groups reduce its stability. The relative stability of the aromatic diazonium cation can also be attributed to the non-localization of the positive charge on nitrogen in the pi (π) system of the ring [11]. Diazonium salts have many important applications, the most important of which is that they are intermediate compounds of organic synthesis because they have the electrophilic property. This is why they are combined with many compounds of large electronic density. Chehim reported the important applications of azo dyes for this purpose [12]. It was found that the stability of complexes is affected by various factors such as the nature of the electron-donating molecule, the bond and the chelating effect, and some parameters such as distribution coefficients, conduction, and refractive coefficients [13].

The aim of the current study was to focused on calculating of the stability constants values of the resulting complexes, which possible to know information related to the quantity or concentration of complexes in the solution. These calculations are very important in many fields of science such as chemistry, biology, and medicine.

In order to approach the goal of the study, the following steps were followed:

1- Preparing and identifying several Schiff bases by chemical and physical methods due to their industrial and medical importance and biological efficacy.

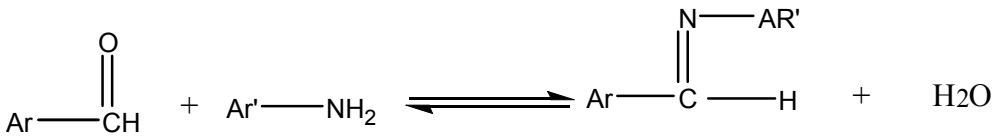
2- Determining the stability constants of the complexes under study after their interaction with the diazotized dinitro aniline reagent.
3- Calculating the thermodynamic parameters for the complexes prepared in different acidic functions (pH) and absolute temperatures, as well as the effect of the type and location of the substituents on the stability constant values.

MATERIALS AND METHODS

Chemicals:
The chemicals used during the research were equipped from the companies (Fluka, BDH, PRS and Nineveh pharmaceutical factory) are: dinitro aniline, sodium carbonate Na_2CO_3, HCl, NaOH, ethanol, Anilin, Aminobenzo phenon, Amino aceto phenon, 4-Amino Syilsilac acid, 5-Amino Syilsilac acid.

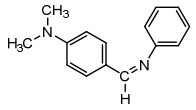
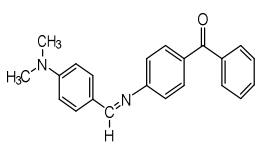
Methods of preparation of compounds:

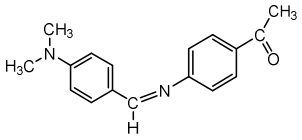
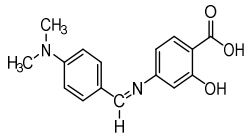
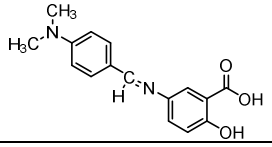
Preparation of Schiff bases: Schiff bases were prepared according Elmali *et al.,* [4], Al-Niemi [5] and Cimerman *et al.,* [14]. Briefly; mixing equal molar amounts of (0.01mole) aldehyde with (0.01mole) of the aromatic primary amine that was dissolved in the least possible ethanol to form an isotropic solution with the resulting water, as shown by the equation:



Each of 4-(diethylamino) benzaldehyde was mixed with the primary aromatic amine in a volumetric flask (100 ml) and the mixture was dissolved with a small amount of ethanol, escalation of the reactant mixture for 2 and half hours, the product was filtered while it was hot after removing the boiling stone. The mixtures were bringing to cold and filtered. The precipitate is taken and a recrystallization process is performed on it to get rid of impurities to obtain pure crystals. The mentioned process is done with an appropriate solvent [15]. Appropriate weights were taken from each of the prepared compounds (Schiff bases) to prepare (10-3M) by transferring them to a beaker and dissolving each of them with ethanol, then transferring to a volumetric vial with a capacity of (200) ml and completing the volume to the mark and then using it in preparing complexes Colored azo and set stability constants and optimal conditions as in table (1), which shows the symbols, names, structural formulas and molecular weights of the prepared Schiff bases.

Table (1): Numbers, names, symbols and some physical properties of the eight aromatic imines compounds with their structures

Comp. No.	Symbol of Derivative	Name	Structure.	M.WT
1	4-DMAB-An	4-(Dimetheylamino) Benzylidne-Anilin		224.3
2	4-DMAB-ABPh	4-(Dimetheylamino) Benzylidne-Aminobenzo phenon		328.4

3	4-DMAB-AAPh	4-(Dimetheylamino) Benzylidne-Amino aceto phenon		266.3
4	4-DMAB-4-ASA	4-(Dimetheylamino) Benzylidne-4-Amino Syilsilac acid		284.3
5	4-DMAB-5-ASA	4-(Dimetheylamino) Benzylidne-5-Amino Syilsilac acid		284.3

Preparation of solutions:

Preparation of the diazotized reagent solutions (DDNA):

A dinitro aniline (0.549 g) is taken and dissolved in a beaker containing (50 ml) of acetonitrile and the solution is placed in a 100 ml volumetric flask and the volume is completed with distilled water up to the mark so that the solvent ratio becomes (1:1) of acetonitrile and distilled water and kept in a cool dark place, using the instant nitrogen method [16] and was applied spontaneously each time.

The diazotized reagent solutions (DDNA) were prepared according to Al-Niemi [5].

The solutions (0.1M) of sodium carbonate as a base and (2N) of hydrochloric acid by standard methods, and these solutions are used to adjust the acidic functions of colored azo imine complexes at the values required for the search and described in table (1).

An amount of (10^{-3}M) of the imine's complexes under study prepared by dissolving appropriate weight from imines complexes in a volume bottle (200ml) and completed to the point of the labeled mark with distilled water.

Instruments:

1- The double beam spectrophotometer on the PG UK-made computer (V5.2.0.1104), production (2012) to check the (λ_{max}) value of the organic complexes under study or the reagents used, and to draw the various electronic spectra with water solvent and in the range between (190-1100nm).

2- Water bath (Memmert comp.) Made (Searle) company Model (L200) for temperature regulation.

3- Balance: A sensitive balance used with four decimal degrees (Julabo sw-A6).

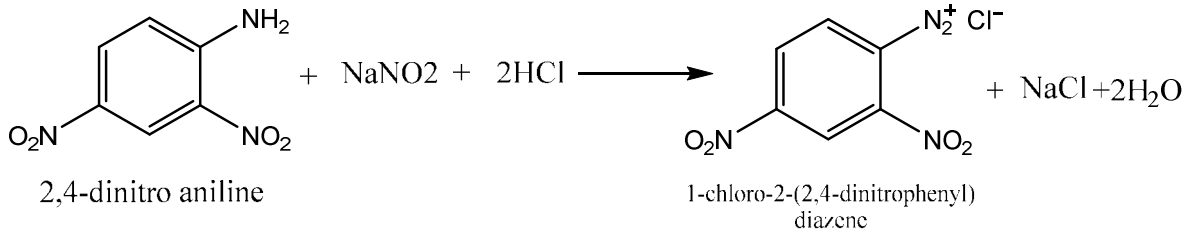
4- Melting point measurement Instrument (30 SMP) production (2003) by (Bibby Scientific Limited) company.

RESULTS AND DISCUSSION

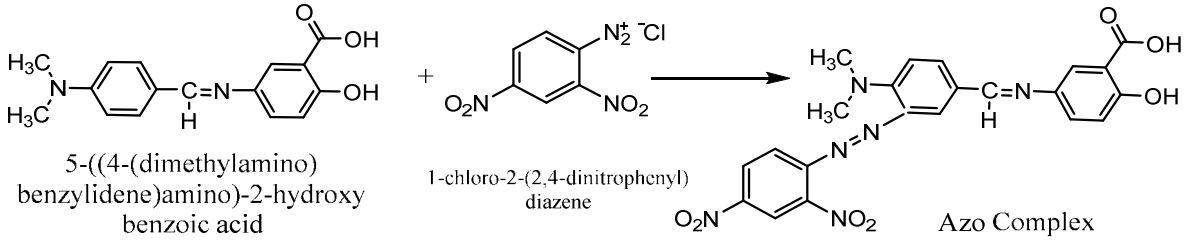
The current study was designated to study the stability constants of azo dyes complexes prepared from the reaction of Schiff bases (aromatic amines that were prepared and diagnosed) with diazotized dinitroaniline (DDNA) reagent, and found the optimal conditions for reactions, the factors that affect the stability constants, and the ratios of the components (stoichiometric ratio) of the complex according to Al-Niemi [5] and Al-Wondawi [15] and Mansoor *et al*. [9]. This study reveals that the stoichiometric ratio of all complexes is (1:1), as well as to be explained in the calculations later.

Preparation of aromatic azoimine dye complexes:

The complex was prepared according to Al-Niemi [5] as shown in the following equation:



The coupling reaction of the denatured reagent (DDNA) occurs with the prepared compounds (Schiff bases) by means of the azo group [17].



Identification methods of the prepared compounds:

1- Chemical methods: Positive and negative detections were used to detected the chemical reactions of complexes, no black precipitation was formed with Tolen and positively with aldehyde.

2- Physical Methods: Melting points were measured for all compounds under study and as shown in the table (2).

Table (2): Some physical properties of the prepared Schiff bases

No.	Symbol of Schiff base	The name	The color	Melting point (°C)
1	4-DMAB-An	4-(Dimetheylamino) Benzylidine- Aniline	Light yellow	71-75
2	4-DMAB-4-ABPh	4-(Dimetheylamino) Benzylidne-4-Aminobenzo phenon	yellow orange	159 -165
3	4-DMAB-4-AAPh	4-(Dimetheylamino) Benzylidne-4-Amino aceto phenon	yellowish orange	122-127
4	4-DMAB-4-ASA	4-(Dimetheylamino) Benzylidne-4-Amino Syilsilac acid	light red	139-142
5	4-DMAB-5-ASA	4-(Dimetheylamino) Benzylidne-5-Amino Syilsilac acid	Brown	135-139 C□

Identification of prepared compounds by (UV and Visible):

The prepared compounds were also diagnosed by UV and visible light (Fig. 1 and table 3).

Table (3) reveals the wavelengths in nanometers for the reactants and products measured with an electronic spectrometer (UV and Visible).

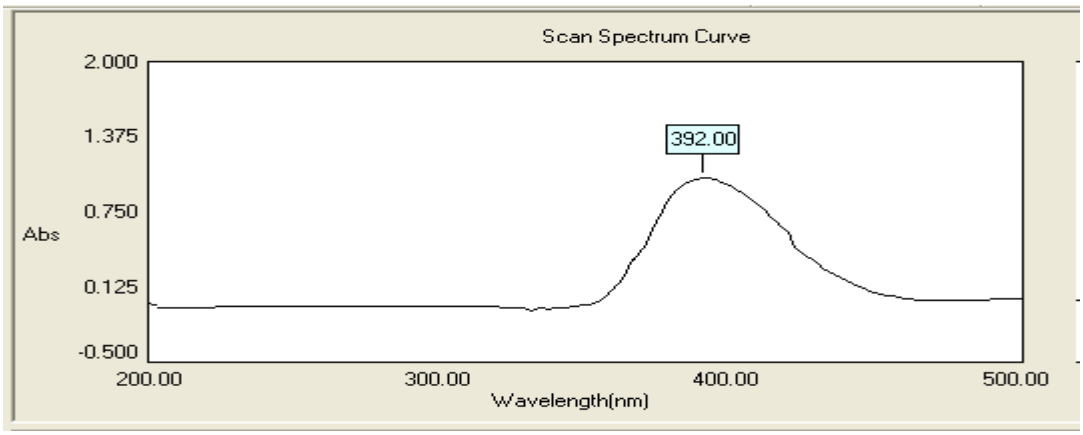


Figure (1): Absorbance spectrum (UV-Vis.) for aqueous solution (10^{-3}M) of (DDNA) reagent

Table (3):The obtained wavelengths of the reactants and products are measured by an electronic spectrophotometer (UV and Visible).

No.	The Symbol	λ_{max}(nm.) for Schiff base	λ_{max}(nm.) (nm.) for Azo compound	Abs. of Azo Complex	λ_{max}(nm.) (nm.) for (DDNA) Reagent
1	4-DMAB-An	314	344	2.022	302
2	4-DMAB-4-ABPh	336	392	1.035	
3	4-DMAB-4-AcPh	328	370	1.831	
4	4-DMAB-4-ASA	326	366	1.712	
5	4-DMAB-5-ASA	320	358	1.980	

The conclusions drawn from table (3) indicate the followings:

1-From the values of wavelengths (λ_{max}) obtained for the complexes and solutions of Schiff bases and for the reagent, it is clear that there is no interference between them, as well as the emergence of new bands for complexes that differ from the bands of the prepared Schiff base solutions and the reagent band.

2-The appearance of the resulting azo complexes in colors that differ from the colors of the Schiff bases under study is another evidence of the formation of new complexes resulting from the interaction of Schiff bases with the diazotized dinitroaniline reagent (DDNA).

Identification of prepared compounds by (IR):

Identification of prepared compounds were demonstrated according to Anderegg *et al.,* [18]. All the IR spectroscopic measurements of all the prepared compounds were measured in the solid state, as shown in the following figures for the IR spectra, starting with the basic compound with aniline (4-DMAB-An) (Table 4 and Fig 2, 3).

All the bands obtained from the infrared spectrum (IR) of all the prepared compounds were tabulated to ensure their identification as shown in table (4).

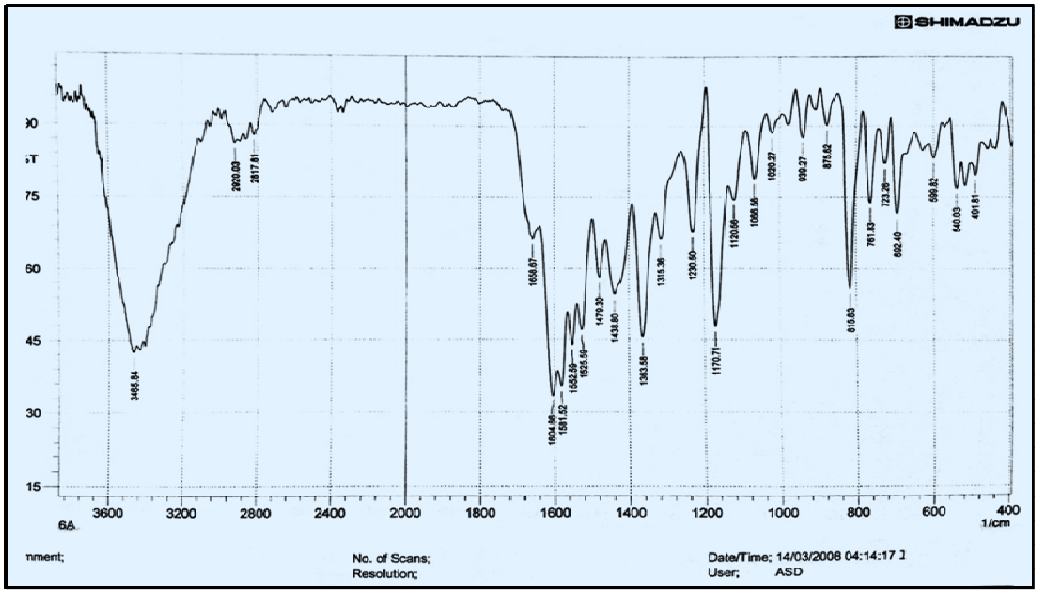


Figure (2): IR spectrum of the compound (4-DMAB-An) in the solid state

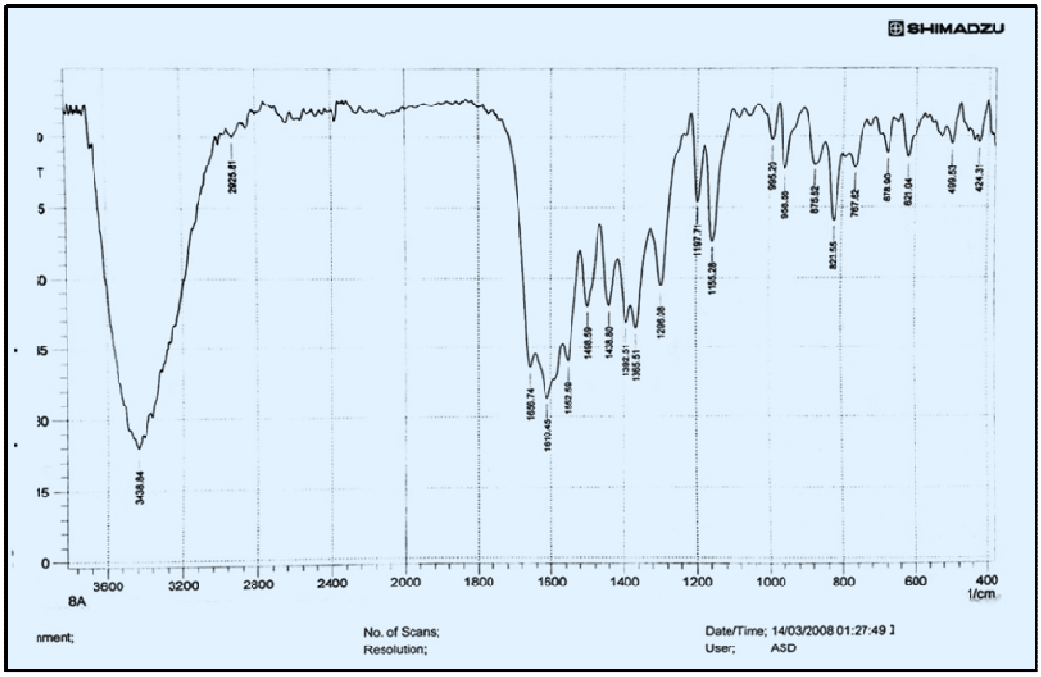


Figure (3): IR spectrum of the compound (4-DMAB-o-T) in the solid state

Table (4): Spectral bands obtained from the infrared spectrum (IR) of all the prepared compounds

NO.	Symbol of comp. Derivatives	c=o υ	c-o υ	C=N υ	ph υ	OH FREE υ	Hb υ
1	4-DMAB-An	-	1479.30 1438.8	1604.66	-	-	346.84
2	4-DMAB-5-ASA	1662.51	1417.58 1502.44	1604.661	1579.59	-	3268.40
3	4-DMAB-4-ASA	1656.74	1438.8	1610.45	1552.54	3438.84	3438.84
7	4-DMAB-4-ABPh	1633.59	1438.8	-	1589.23	3417.63 3332.76	3332.76
8	4-DMAB-4-AcPh	1666.3	-	-	1577	-	3319.26

Study of the optimum conditions for the prepared azoimine complexes:

After the four complexes under study were diagnosed by several methods, including chemical and physical ones available in our laboratories, studying the optimum conditions for the formation of complexes of azo dyes after reacting the prepared Schiff bases was done with the diazotized dinitroaniline (DDNA) reagent, which is fresh azotization at a temperature of (273-278^{o}K). The most important optimal conditions are:

1-The optimum wavelength (λ_{max}).

2-The optimum volume for the used reagent (DDNA).

3-The optimum volume of (Na_2CO_3).

4-The optimum order of addition.

5-The optimum appearance time for the complexes formed was zero minutes in the complexes studied.

6-The optimum stability time for the formed complexes.

7-The optimum temperature for the formation of complexes.

The optimum conditions for each azo-imine dye formed by the reaction of the prepared imines with the diazotized reagent (DDNA) at certain pH and temperature (283^{o}K) were studied and carefully tabulated to avoid repetition in speech, as in the following table (5).

Table (5): Optimum conditions for the four azo-imine dyes produced at certain pH and temperature (283^{o}K)

No.	Symbol of Complex	pH	Optimum Order of Addition	Optimum Wavelength(nm.)	Appearance time(min)	stability time(min)
1	4-DMAB-An	7.6	0.2Sch. + 1.5R + 0.2B	344	0	35
		9.2	0.25B + 1.5R + 0.2 Sch.	338	0	40
2	4-DMAB-4-ASA	7.4	0.3R + 0.2B + 0.4D	366	0	30
		9	0.3R + 0.5B + 0.4D	360	0	40
3	4-DMAB-5-ASA	7.2	0.5R + 0.2B + 0.2D	358	0	40
		9	0.2D + 0.9B + 0.5R	350	0	45
4	4-DMAB-4-ACPh	7	0.4R + 0.3B + 0.4D	370	0	55
		9	0.4R + 0.5B + 0.4D	362	0	75
5	4-DMAB-4-ABPh	7.2	0.3B + 0.3R + 0.2D	392	0	25
		9	0.9B + 0.3R + 0.2D	378	0	40

Throughout table (5), the following notes had been revealed:

1- The structural form and the acidic functions (pH) are the most important factors in determining the optimal conditions and consequently the order of addition. This is in agreement with previous studies [5,19, 20].

2- The wavelength (λ_{max}) in solutions with an acidic medium is higher than that of the same compound at a neutral or basic medium for most azo compounds, and the reason is due to the partial protonation that occurs in the azo dye complex or the formation of the nitrillium ion in the acidic medium, which is stable by resonance, which gives ease Due to the movement of electrons in the complex and thus the absorption energy of the complex decreases, and also due to the implicit hydrogen bond in the complex that forms an additional stable hexagonal ring for azo dye complexes [19-21].

Finding the ratios of the components (Stoichiometric ratio) for complexes:

Several studies had confirmed that the photometric method includes in its aspects [3,19] different methods, namely the job method, the molar ratio and the slope ratio method. Another study [22] showed that the molar ratio method is more Common among those methods because they can be applied to different systems, and the molar ratio method was adopted in our study.

When drawing a graphic relationship between the absorbance against the molar ratio (of the reagent/imine) a direct relationship is produced between them for all the resulting complexes at a constant temperature (283^{o}K). This relationship continues for the four compounds under study at certain acidic functions until reaching the maximum values that represent the ratios of the components of the process complex. It is also noticed after that the transformation of the relationship referred to above into an inverse relationship or the occurrence of some kind of negative deviation. Table (6) shows the mole ratios of the four donors prepared with the reagent (DDNA) at certain acid functions and at a temperature of (283^{o}K).

Table (6): The mole ratios of the four donors prepared with the reagent (DDNA) at certain (pH) and at a temperature of (283^{o}K)

No.	Symbol of Complex	pH	Optimum Order of Addition	Optimum Wavelength(nm.)	Appearance time(min)	stability time(min)
1	4-DMAB-An	7.6	0.2Sch. + 1.5R + 0.2B	344	0	35
		9.2	0.25B + 1.5R + 0.2 Sch.	338	0	40
2	4-DMAB-4-ASA	7.4	0.3R + 0.2B + 0.4D	366	0	30
		9	0.3R + 0.5B + 0.4D	360	0	40
3	4-DMAB-5-ASA	7.2	0.5R + 0.2B + 0.2D	358	0	40
		9	0.2D + 0.9B + 0.5R	350	0	45
4	4-DMAB-4-ACPh	7	0.4R + 0.3B + 0.4D	370	0	55
		9	0.4R + 0.5B + 0.4D	362	0	75
5	4-DMAB-4-ABPh	7.2	0.3B + 0.3R + 0.2D	392	0	25
		9	0.9B + 0.3R + 0.2D	378	0	40

From the previous table (6), we note that the molar ratios of all the prepared compounds are confined between (1 - 1.2), and this means that the mole ratios of all the prepared compounds are (1:1) in all the specific acidic functions (pH), and this is consistent with a previous study [23].

Calculation of the stability constants for the prepared azoimine complexes:

The stability constants of the compounds resulting from the reaction of the four prepared imines with (DDNA) reagent were calculated at different pH functions and temperatures (273, 283, 293, 303 and 313^{o}K). The obtained results are tabulated as in table (7), whereas:

Es= absorption of a solution of the resulting compound containing stoichiometric ratios of reagent and imine (Schiff's base) prepared. Under these conditions, the formed azo compound is relatively dissociated, with little absorption (Es).

Em= absorption of a solution of the resulting compound containing excess of the reagent, under optimal conditions. The solution is prepared in the same way as the first solution and is similar to it, but it contains an excess of reagent and at optimal conditions according to the table (Optimal Conditions). Here the resulting compound is more stable and the absorbance value is higher (Em).

After that, we find the product of (Em-Es)/Em for each studied solution against the blank solution in order to find the value (α) from which the stability constant (K) is calculated, as follows: $K=(1-\alpha)/(\alpha^2.C)$.

Table (7): The values of the stability constants for all aromatic azoimine complexes in different (pH) and temperatures prepared according to the optimal conditions and at their stability times

NO.	Symbol of Azo complexes	PH	T(K)	Es	Em	ȧ	K
1	4-DMAB+An	7.6	273	0.891	1.309	0.31933	333760
			283	0.571	0.882	0.35261	260348
			293	0.697	1.085	0.3576	251171
			303	0.567	0.928	0.38901	201877
			313	0.503	0.906	0.44481	140299
		9.2	273	0.815	1.053	0.22602	757534
			283	0.608	0.828	0.2657	520066
			293	0.592	0.903	0.34441	276350
			303	0.584	0.912	0.35965	247531
			313	0.579	0.946	0.38795	203333
2	4-DMAB+5-ASA	7.2	273	0.885	0.979	0.09602	4902756
			283	0.817	0.938	0.129	2617123
			293	0.792	0.931	0.1493	1908162
			303	0.756	0.917	0.17557	1337240
			313	0.709	0.991	0.28456	441765
		9	273	0.547	1.345	0.59331	57766.2
			283	0.571	1.489	0.61652	50444.7
			293	0.497	1.409	0.64727	42096.7
			303	0.491	1.447	0.66068	38869.1
			313	0.395	1.257	0.68576	33410.9

NO.	Symbol of Azo complexes	PH	T(K)	Es	Em	ȧ	K
3	4-DMAB+4-Acph	7	273	0.815	0.954	0.1457	2012085
			283	0.625	0.758	0.17546	1339109
			293	0.608	0.745	0.18389	1206671
			303	0.6	0.819	0.2674	512291
			313	0.606	0.832	0.27163	493570
		0	273	0.723	0.893	0.19037	1117022
			283	0.861	1.082	0.20425	953709
			293	0.848	1.116	0.24014	658810
			303	0.802	1.071	0.25117	593512
			313	0.773	1.043	0.25887	552976
4	4-DMAB+4-ASA	7.4	273	0.873	0.984	0.1128	3486048
			283	0.816	0.935	0.12727	2693878
			293	0.774	0.89	0.13034	2559676
			303	0.736	0.864	0.14815	1940625
			313	0.701	0.851	0.17626	1325669
		9	273	0.807	0.96	0.15938	1654748
			283	0.753	0.902	0.16519	1529674
			293	0.729	0.886	0.1772	1310183
			303	0.7	0.858	0.18415	1202932
			313	0.654	0.828	0.21014	894293
5	4-DMAB+4-ABph	7.2	273	0.721	0.831	0.13237	2475831
			283	0.709	0.819	0.13431	2399467
			293	0.693	0.811	0.1455	2018181
			303	0.584	0.685	0.14745	1960788
			313	0.517	0.627	0.17544	1339500
		9	273	0.876	0.947	0.07497	8228248
			283	0.801	0.925	0.13405	2409355
			293	0.781	0.948	0.17616	1327384
			303	0.687	0.839	0.18117	1247388
			313	0.565	0.694	0.18588	23562.9

From table (7), we conclude the following:

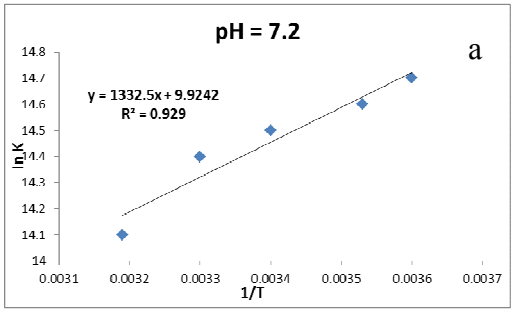
1-The values of the stability constants of the azo-imine dye compound generally decrease with increasing temperatures. This is consistent with the increasing degree of dissociation (α).

2-The values of the stability constants for the azo-imine dye compound formed vary according to the values of the acid functions. The reason is due to the fact that the electron pairs present on the active groups in Schiff bases are more likely to share with the reagent (DDNA) in the base medium than the acidic medium, and that the presence of these compounds in the medium works to restrict the electronegative pairs in the active groups, thus becoming less amenable to electronic donating and thus be weaker Lewis's base.

3-Five different values of stability constants appeared for the azoimine dye compound formed at five different temperatures and for all the azo compounds formed. This means that these complexes are stable at these five temperatures.
4-The difference in the values of the stability constants for the formed four azo-imine dye compounds at the same temperature and at the same acid function is evidence of the effect of the structure of Schiff base on the values of the stability constants.

Thermodynamic study for the prepared azoimine complexes:

The stability study of all four azo-imine compounds formed at the optimal conditions for each of them, at different temperatures with a range of (273-313^{o}K) and at different acid functions (pH), we were able to find the thermodynamic parameters for their formation.
This study showed the effect of temperature on the values of the stability constants of the azo-imine compounds formed through the direct relationship obtained from the graphs between (InK) against (1/T), as illustrated in figures (6-a, 6-b, 7-a and 7-b).



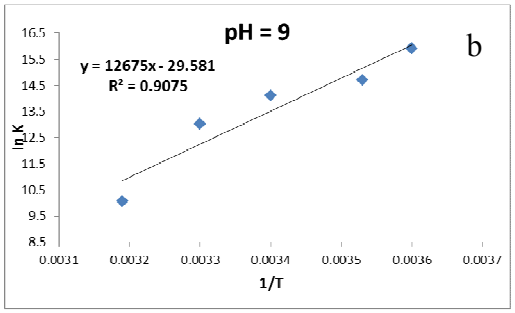
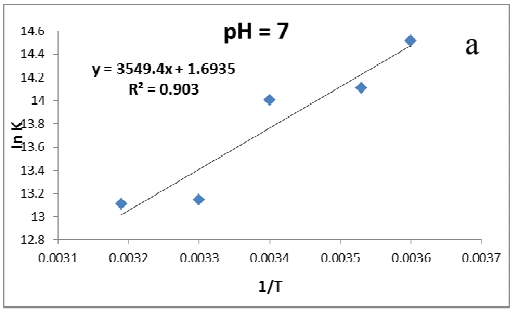


Figure (6-a, b): The effect of absolute temperatures on the stability constants of the azo-imine compound formed (4-DMAB-4-ABph-DDNA) under optimal conditions and at different acid functions (pH).



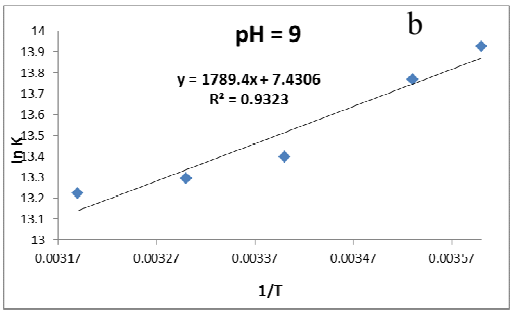


Figure (7-a,b): The effect of absolute temperatures on the stability constants of the azo-imine compound formed (4-DMAB-4ACph-DDNA) under optimal conditions and at different acid functions (pH).

The values of enthalpy change (ΔH) were calculated from the mathematical relationship (ΔH=-R*Slope). It was negative for all the resulting compounds and at all temperatures and pH functions. These negative values indicate that these formation reactions are exothermic, and therefore the values of the stability constants decrease and the degrees of dissociation increase when temperatures rise.
The values of change in the standard Gibbs free energy (ΔG) were calculated from the mathematical relationship (ΔG=-RT ln) . It was negative for all the resulting compounds and at all temperatures and pH function. These negative values indicate that these formation reactions are spontaneous towards the products.
The values of standard entropy change (ΔS) were calculated from the mathematical relationship (ΔS=(ΔH-ΔG)/T). It was negative for all the resulting compounds and at all temperatures and pH functions. These negative values indicate that these formation reactions reduce the randomness of the products.
The calculations mentioned above have been tabulated in the following table (8), which gives a summary of the thermodynamic study of the four resulting compounds at all temperatures and pH functions.

Table (8): A summary of the thermodynamic study of the four resulting compounds at all temperatures and pH functions

NO.	Symbol of Azo complexes	PH	T(K)	ln k	ΔḢ□	ΔG□	ΔS□	ΔHAV.	ΔGAV.	ΔSAV.
1	4-DMAB+ANILINE	7.6	273	12.7182	-15.561	-28.867	0.04874	-15.561	-30.022	0.04934
			283	12.4698	-15.561	-29.34	0.04869			
			293	12.4339	-15.561	-30.289	0.05027			
			303	12.2154	-15.561	-30.772	0.0502			
			313	11.8515	-15.561	-30.841	0.04882			
		9.2	273	13.5378	-26.566	-30.727	0.01524	-26.566	-31.062	0.01532
			283	13.1617	-26.566	-30.968	0.01555			
			293	12.5294	-26.566	-30.522	0.0135			
			303	12.4193	-26.566	-31.286	0.01558			
			313	12.2226	-26.566	-31.807	0.01674			
2	4-DMAB+5-ASA	7.2	273	15.4053	-43.088	-34.966	-0.0298	-43.088	-34.865	-0.0281
			283	14.7776	-43.088	-34.77	-0.0294			
			293	14.4617	-43.088	-35.229	-0.0268			
			303	14.1061	-43.088	-35.535	-0.0249			
			313	12.9985	-43.088	-33.826	-0.0296			
		9	273	10.9642	-10.687	-24.886	0.05201	-10.687	-26.006	0.05228
			283	10.8286	-10.687	-25.478	0.05227			
			293	10.6477	-10.687	-25.938	0.05205			
			303	10.568	-10.687	-26.622	0.05259			
			313	10.4166	-10.687	-27.107	0.05246			
6	4-DMAB+4-Aac.ph	7	273	14.5147	-29.51	-32.944	0.01258	-29.51	-33.496	0.01359
			283	14.1075	-29.51	-33.193	0.01302			
			293	14.0034	-29.51	-34.112	0.01571			
			303	13.1466	-29.51	-33.118	0.01191			
			313	13.1094	-29.51	-34.114	0.01471			
		9	273	13.9262	-14.877	-31.609	0.06129	-14.877	-32.908	0.06153
			283	13.7681	-14.877	-32.394	0.0619			
			293	13.3982	-14.877	-32.638	0.06062			
			303	13.2938	-14.877	-33.489	0.06143			
			313	13.2231	-14.877	-34.41	0.06241			
7	4-DMAB+4-ASA	7.4	273	15.0643	-17.735	-34.192	0.06028	-17.735	-35.626	0.06105
			283	14.8065	-17.735	-34.838	0.06043			
			293	14.7554	-17.735	-35.944	0.06215			
			303	14.4785	-17.735	-36.473	0.06184			
			313	14.0974	-17.735	-36.685	0.06054			

NO.	Symbol of Azo complexes	PH	T(K)	ln k	ΔḢ□	ΔG□	ΔS□	ΔHAV.	ΔGAV.	ΔSAV.
		9	273	14.3192	-11.692	-32.501	0.07622	-11.692	-34.25	0.07698
			283	14.2406	-11.692	-33.506	0.07708			
			293	14.0857	-11.692	-34.313	0.0772			
			303	14.0003	-11.692	-35.269	0.07781			
			313	13.7038	-11.692	-35.661	0.07658			
8	4-DMAB+4-Ab.ph	7.2	273	14.7221	-11.078	-33.415	0.08182	-11.078	-35.312	0.0827
			283	14.6908	-11.078	-34.565	0.08299			
			293	14.5177	-11.078	-35.365	0.08289			
			303	14.4889	-11.078	-36.499	0.0839			
			313	14.1078	-11.078	-36.712	0.0819			
		9	273	15.9231	-105.38	-36.141	-0.2536	-105.38	-33.324	-0.2461
			283	14.6949	-105.38	-34.575	-0.2502			
			293	14.0987	-105.38	-34.345	-0.2424			
			303	14.0366	-105.38	-35.36	-0.2311			
			313	10.0674	-105.38	-26.198	-0.253			

From table (8), we conclude the following:

1-Negative (ΔH) and ($\Delta H_{av.}$) values, which gave stability constants for the four azoimine compounds produced at all five temperatures and at the acidic functions shown, indicating that the formation reactions of azoimine compounds are exothermic reactions.

2-The values of (ΔG^o) and ($\Delta G^o_{av.}$) for the above-mentioned reactions were also negative for the four azoimine compounds produced at all five temperatures and at the acid functions shown. This indicates that the reaction of its formation is a spontaneous reaction.

3-As for the values of the change in entropy (ΔS^o), they should be negative in theory. This is consistent with most of the studied compounds at certain acidic functions. As for the compounds that gave positive (ΔS^o) values, the reason is attributed to the action of the hydrogen bond in these complexes whose strength and energy are affected by temperature change, in other words, the hydrogen bonds transform change values of (ΔS^o) to the positive sign.

4-The different values of (ΔS^o, ΔG^o, ΔH) when the resulting compound differs is believed to be caused by the structures of the studied complexes. This is consistent with a lot of studies (Elena P. Salvador, 2012).

REFERENCES

1. Moldoveanu SC. (2019). Pyrolysis of Organic Molecules. 2nd Edition, PP: 123-144.

2. Cimerman Z.; Milijanic S. and Galic N. (2000). Schiff bases derived from aminopyridines as spectrofluorimetric analytical reagents. *Croatica Chemica Acta.* 73(1): 81-95.

3. Patel PR.; Thaker BT. and Zele S. (1999). Preparation and characterization of some lanthanide complexes involving a heterocyclic~-diketone. *Ind. J. Chem.* 38:563-567.

4. Elmali A.; Kabak M. and Elerman Y. (2000). The rapid synthesis of Schiff's bases without solvent under microwave irradiation. *J. Molec. Struct.* 477: 151-159

5. Al-Niemi MM. (2005). A Comparative Study on The Factors Affecting on Stability Constants Values of Some Azo Imines Phenolic Dyes. Ph. D. Thesis, University of Mosul.

6. Moldoveanu SC. (2019). Pyrolysis of Organic Molecules Application to Health and Environmental issues (2nd Edition), Elsevier Science. PP.: 123-144.

7.Quach DL.; Mincher BJ. and Wai CM. (2014). Supercritical fluid extraction and separation of uranium from other actinides. *J.Haz.Mater.* 274:360-366.

8. Sharma SK.; Rizwana M. Ali S.; Shahzadi S.; Qanungo MS. and Mahmood S. (2013). Synthesis,

characterization, HOMO-LUMO study and antimicrobial activity of organotin (IV) complexes of 4-piperidine carboxamide its Schiff base. *J. Coord. Chem* 31(5): 141-146.

9. Malik MA.; Ahmad Dar O.; Gull P.; Wani MY. and Hashmi AD. (2018). Heterocyclic Schiff base transition metal complexes in antimicrobial and anticancer chemotherapy. *Med. Chem. Commun.* 9: 409-436.

10. Clayden J.; Greevs N. and Warren S. (2012). organic chemistry. USA , 2nd ed. PP: 91-121.

11. Sethi A.; Bhatia A; Shukla D.; Kumar RS. and Prkash GB. (2012). Synthesis of diosgenin *p*-nitrobenzoate by Steglich method, its crystal structure and quantum chemical studies. *J. Molec. Struct.* 1028, 88-96.

12. Chehim MM. (2012). New Coupling Agents in Polymer and Surface Science. In Aryl Diazonium Salts. Eds. McCreen and Bergren.

13. Singh J.; Srivastav AN.; Singh N. and Singh A. (2020). Stability Constants of Metal Complexes in Solution. PP: 621-652.

14. Cimerman Z.; Miljanić S. and Galić N. (2000). Schiff bases derived from aminopyridines as spectrofluorimetric analytical reagents. *Croatica Chemica Acta.* 73(1):81-95.

15. Al-Wundawi AQ. (2020). A spectroscopic and theoretical study of the stability of a number of imine complexes resulting from the reaction of benzylidene-aniline with diazotized para-nitroaniline reagent. Master thesis, University of Mosul.

16. Mwene-Mbeia TM. (2019). Chemical Stability of Pharmaceutical Organic Compounds. Am. J. Biomed Sci. Res. 6(1): 14- 22

17. Sarkis JY.; Al-Habib MJ.; Al-Hassan RR. and Hussein FA. (1990). The Foundations of Organic Chemistry. Master Thesis. Al-Mustansiriya University.

18. Salvador EP. (2012). Thermodynamic aspects of selective complexation of heavy metal ions of environmental interest: fundamentals and applications. Ph. D. thesis. Bellaterra, Autonomous University of Barcelona. PP: 32-33.

19. Azzouz ASB. and Agha AN. (2005). The Influence of Surfactants and Solvent on the Stability Constant Value of some Azo Dyes Formation Between Oxime and the Diazotized sulphanpilin Acid Salt. *J. Edu. Sci.,* 17: 11-15.

20. Azzouz ASB. and Al-Niemi MMH. (2005). Study on association of substituted benzoic acid and other acids by physical. Effect of temperature and structure of acids on association process. *Z. Phys. Chem.* 219, 1591-1608.

21. Azzouz ASB.; Abdulla KA. and Al-Niemi I. (1995). The role of hydrogen bonding on kinetics of rearrangement of heterocyclic aldoximes in perchloric acid. *Mu'tah Lil-Buhooth Wa Al-Dirasat* 10 (1), 77-91.

22. Giunta CJ. (2016). Amount of Substance, Chemical Amount, and Stoichiometric Amount. *J. Chem. Educ.* 93(4): 583–586.

23. Mabrouk M.; Hammad SF.; Abdelaziz MA. and Mansour FR. (2018). Ligand exchange, method for degermation of mole ration of relatively weak metal. *Chem. Centr. J.* 12:143.

24. Batsanov SS. and Batsanov AS. (2012). Introduction to Structural Chemistry. Springer, USA. P. 264.

Potential source of antibiotics extracted from Jordanian soil microbes against MRSA and *Acinetobacter baumannii*

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ABSTRACT

Jordan possesses a unique diversity of natural native soil, which has a microbial population that is not well understood. Streptomyces and Bacillus species were isolated from the unique location from Jordanian soil and show antimicrobial activities. The current study aimed to evaluate the bioactive extracts from these microorganisms against hospital-resistant bacteria. The bioactive substances were extracted from four bacillus and three Streptomyces species by n-butanol and their antimicrobial activity on nine clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) and 2 *Acinetobacter baumannii*were collected from Islamic Hospital in Amman, Jordan. Antibacterial activity for the soil isolates was determined essentially by a standard cup-plate method. Briefly; the plates were swabbed with the clinical isolate strains, the dried n-butanol extracts were dissolved in 100 µl of corresponding solvents and then the butanol evaporates extract were filled up in the well's plates of solid agar media. All samples were used in duplicates to confirm the results. Zone of inhibition was measured after 24 - 48 h incubation at 37 0 C. The antimicrobial activity was estimated by measuring the diameter of the inhibitory zone. N-butanol solution was used as a negative control. Results showed that the Streptomyces and Bacillus isolated strains used in the current study have excellent and significant antibacterial activity against Methicillin-Resistant *Staphylococcus aureus* (MRSA), *and Acinetobacter baumannii.* Two major soil isolates that showed the highest activity were *Bacillus spp.* KTD 119 and *Streptomycetes spp.,* NF 141 with a maximum zone of inhibition (42 and 30 mm) on MRSA *and Acinetobacter baumannii* respectively. We conclude that the *Streptomycetes spp.,* and *Bacillus spp.,* strains isolated from unique Jordanian soil have excellent activities against MRSA *and Acinetobacter baumannii.* Also concluded that the n-butanol substances extracted from the seven bacteria belonged to *Streptomycetes spp.,* and *Bacillus spp.* strains represent a promising source of antibacterial agents against resistant bacteria. This also brings to light the importance of documentation of an antibiotic sensitivity profile of pathogenic isolates as an essential clinical procedure to combat and evaluate the problem of antibiotic resistance for effective treatment.

Keywords: Soil Microorganisms, Resistant bacteria, MRSA, Acinetobacter.

INTRODUCTION

Resistant bacteria isolated from patients was rapidly emerging as infectious agents in Jordanian hospital because of abuse of antibiotics, unpleasant and nasty bacteria were reported [1,2]; namely: methicillin-resistant *Staphylococcusaureus* (MRSA), *Acinetobacter baumannii*. Antibiotics resistance among pathogenic bacteria is a worldwide problem that challenges the strategy for fighting and controlling these microorganisms. On other hand, there is a sharp decline in the number of newly approved antibiotics has further complicated the treatment process [3].

This article among project started in 2009 and still working to explore the unique location in Jordan not have explored before to discover a new antimicrobial that fights the resistant bacteria, which hardly treated with known antibacterial agents present in the market. Clinical resistant bacteria were isolated and identified previously [4], included: 9 Methicillin-Resistant Staphylococcus aureus (MRSA), and 2 *Acinetobacter baumanni. Acinetobacter baumannii* is a rapidly emerging pathogen in the health care setting, where it causes infections that include bacteremia, pneumonia, meningitis, urinary tract infection, and wound infection. Antimicrobial resistance among *Acinetobacter* species has increased substantially in the past decade [5,6]. The capacity of *Acinetobacter* species for extensive antimicrobial resistance may be due in part to the organism's relatively impermeable outer membrane and its environmental exposure to a large reservoir of resistance genes. Some strains are susceptible only to polymyxins—peptide antibiotics that are not routinely used because of earlier reports about toxicities [6]. On the other hand, the methicillin-resistant strains of *S. aureus (*MRSA) are the most problematic bacterium in public health not only because it is highly prevalent but also because it has become resistant to almost all available antibiotics except vancomycin and teicoplanin. Recently, its susceptibility to vancomycin has decreased, and vancomycin-intermediate and vancomycin-resistant *S. aureus* have increasingly been found in several countries. Furthermore, a decrease in the susceptibility of MRSA to teicoplanin has also been reported in several hospitals around the world. The evidence of MRSA resistance to vancomycin and teicoplanin, which are antibiotics of last resort, makes the need for alternative antibiotics and chemotherapeutics after vancomycin and teicoplanin treatments have failed particularly urgent [6]. The current study aimed to evaluate the bioactive substances' extracts by n-butanol method from four bacillus and three Streptomyces species and their antimicrobial activity against clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA)*, and Acinetobacter baumannii.*

MATERIALS AND METHODS

Clinical isolates:

Nine Methicillin-Resistant Staphylococcus aureus (MRSA), and two Acinetobacter *baumanni* were described and characterizes in the previous study [4].

Soil bacteria:

Soil bacteria were isolated from Jordanian soil and fully characterized previously [7]. These included 3 Streptomyces's species and 4 Bacillus specie's (Table 1).

Table (1): The antibiotic Bacterial species producer used in this study

Tested isolated code	
Bacillus spp.	**KTD 119**
Bacillus spp.	**KTD 120**
Streptomycetes spp.	**KTD 123**
Bacillus spp.	**KTD 133**
Bacillus spp.	**NF 131**
Streptomycetes spp.	**NF 140**
Streptomycetes spp.	**NF 141**

Extraction and purifications of bioactive substances:

The flasks containing producer bacteria were incubated at 28 C for 120 hours with shaking at 105 t/min. After growth, the cell-free culture supernatant of each flask was extracted twice with an equal volume of n-butanol [8-10]. The n-butanol layer was separated from the aqueous phase and concentrated on a rotary vacuum evaporator. The butanol extracts containing a bioactive component of each seven-sample fractionated using thin-layer chromatography (TLC) on a silica gel plate and developed with the solvent system [11]. The TLC band of each sample was scrapped into vials and dissolve by solvent, then the solvent was drying by nitrogen gas, and the precipitates dissolved again with sterile deionized water. The solvent is allowed to completely evaporate off. Bands were scraped

from the plates with a spatula under UV light, extracted with methanol, and filtered through Whatman No. 5 paper. Each band was bio-assayed using *Staphylococcus aureus (S.aureus)* ATCC2923 and *Escherichia coli (E.coli)* ATCC25922, the active bands were purified again on TLC using the same solvent system and visualized under UV light [11]. The *Rf* for each band was measured. Each isolated band was also dissolved in methanol, and its UV absorption spectrum was measured to determine the maximum of the band. Compares between *Rf* value of our samples and *Rf* of references antibiotics that tested on TLC [12].

Assay for antimicrobial activity:

Two methods were used according to nature of testing. The disc diffusion method was used according the Kirby-Bauer test and described by National Committee for Laboratory Standards [13] and cup plate methods [14]. Briefly; the plates were swabbed with the clinical isolate strains, the dried n-butanol extracts were dissolved in 100 µl of corresponding solvents and then the butanol evaporates extract were filled up in the well's plates of solid agar media. All samples were used in duplicates to confirm the results. Zone of inhibition was measured after 24 - 48 h incubation at 37 0 C. Results interpretation depend upon zone of inhibitions observed from back against a black ground illuminated with reflected light to measures the diameter of zones of inhibition to the nearest millimeters. N-butanol without extracts used as a negative control.

Determination of Minimum Inhibitory Concentrations (MICs) of bioactive substances:

To determine the MICs of the seven bioactive substances for the 9 MRSA and 2 *Acinetobacter baumannii*, the broth microdilution method [13] was applied and directed by CLSI M07-A9 guidelines [15]. The concentration of bioactive substances use was 0.0100 ug/ml to 100 ug/ml.

RESULTS

Nine MRSA strains were subjected to n-butanol extracted from *Streptomyces* spp. strain (NF 140, 141 and KTD123) and *Bacillus* species strains (KTD 119, 120, 133 and NF 131, isolated from Jordanian soil. Table (2) reveals that the Bacillus species KTD 119 had superior activity on MRSA than the other bioactive substances extracted from test organisms with the highest zone of inhibition 42 (mm) on both Z100 and E100 followed by Streptomyces NF 141 with the highest zone of inhibition 30 (mm) on D100, U100 and E100 and variable activity by others (Table 2).

Figures 1 and 2 show comparisons of effects of known antimicrobials from our previous study [4], and the bioactive substances extracted and purified by the current study; it's clear that the bioactive substances more potent and efficient.

Table (2): Antimicrobial activity of bioactive substances extracted from Bacillus and Streptomyces species isolated from Jordanian soil against MRSA

Test Organisms	Zone of Inhibition (mm)						
	KTD 119	KTD 120	KTD 123	NF 131	KTD 133	NF 141	NF 140
Y 100	38	20	15	18	zero	26	17
E 100	42	22	12	9	zero	30	23
S 100	26	18	23	18	10	23	20
A 100	20	15	10	26	9	18	16
D 100	26	18	14	24	10	30	27
X 100	22	21	9	22	15	18	13
U 100	16	18	13	9	15	30	24
M 100	14	9	14	20	18	16	15
Z 100	42	20	12	19	16	20	17

Excellent synergism effects on MRSA were observed (Figure 3). The bioactive substances extracted from Bacillus species strains KTD119 and 120 with Streptomyces's species strain NF140.

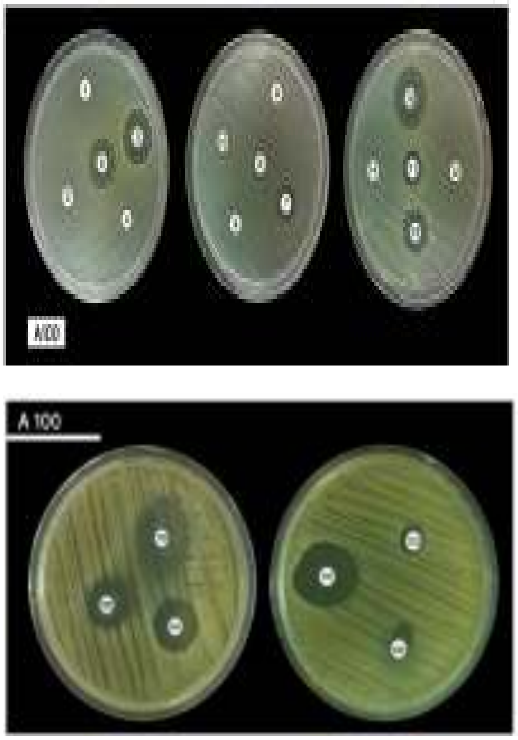

Figure (1): Antibiogram of MRSA strain A100 isolated from clinical specimens. The upper frame shows the antimicrobial resistant activities to; ZOX, FEP, OX, TGC, CL, LEV, CRO, GN, CIP, AK, AMc and sensitive to VA, TEC and SXT. The lower frame shows the activities of bioactive substance extracted from Bacillus species strains (KTD 119, 120, 133 and NF 131) and Streptomyces species strain KTD 123 and NF 141

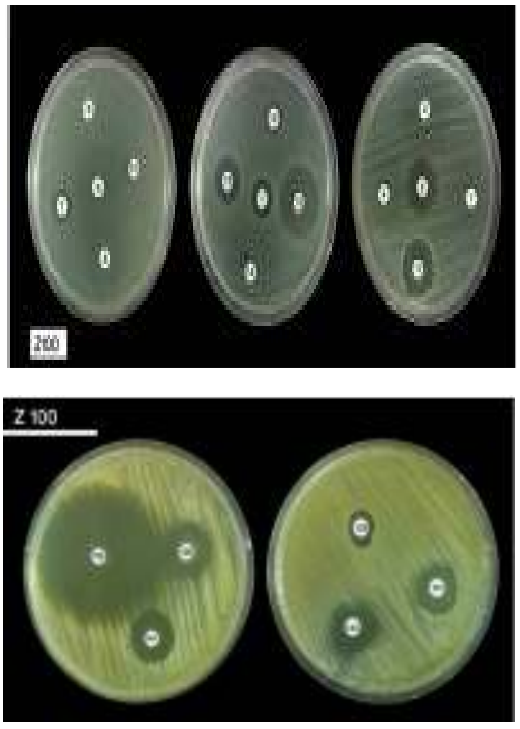

Figure (2): Antibiogram of MRSA strain Z100 isolated from clinical specimens. The upper frame shows the antimicrobial resistant activities to; ZOX, FEP, OX, TGC, CL, LEV, GN, CIP, AK, AMc and sensitive to VA, TEC and SXT. The lower frame shows the activities of bioactive substance extracted from Bacillus species strains (KTD 119, 120, 133 & NF 131) and Streptomyces's species strain KTD 13 and NF141

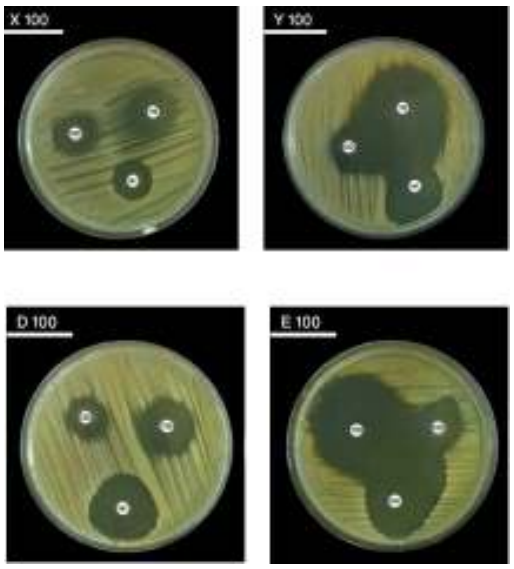

Figure (3): The synergism effects of bioactive substances extracted from Bacillus species strains KTD 119 and 120 with Streptomyces's species strain NF 140 on MRSA strains X100 and Y 1100 (upper frame) and D 100 and E 100 (lower frame).

The seven n-butanol extracts from *Streptomyces* (NF 140 NF 141 and KTD 123) and *Bacillus* species namely (KTD 119, KTD 120, NF 131, and KTD 133) were screened for their antimicrobial activity against *Acinetobacter baumannii* strains A5 and A14. Table (3) shows that Bacillus species strains KTD 119, KTD120 and KTD 123 possess good antimicrobial activities with the highest zone of inhibitions 30 (mm), 30 (mm), and 18 (mm) respectively, while the *Streptomyces species strains* NF 140 and NF 141 does not have any activity.

Table (3): Screening of soil isolates extracts for their antimicrobial activity on *Acinetobacter baumannii* By Cup-Plate Method

Test of organisms	Zone of inhibition						
	KTD 119	KTD 120	KTD 123	NF 131	KTD 133	NF 140	NF 141
A5	30	30	9	0	0	0	0
A14	30	30	18	0	0	0	0

Calibrations for the strains, antibiotics, disc potencies and acceptable zones of inhibition were based on NCCLS standards as given in table (4). A total of two *Acinetobacter baumannii* isolates described as A5 and A14 were tested. Table (4) and figure (4) revealed that all tested *Acinetobacter baumannii* were resistant to all mentioned antimicrobial agents.

Table (4): The disc-Antibiotics susceptibility of *Acinetobacter baumannii* isolates collected from Islamic Hospital – Jordan [4].

Antimicrobial agent	Zone of inhibition (mm)	
	A5	A14
Amoxicillin/Calvulanic acid	Zero (R)	Zero (R)
Sulfamethoxazole/Trimethoprim	Zero (R)	Zero (R)
Amikacin	12(R)	Zero (R)
Gentamicin	Zero (R)	Zero (R)
Ertapenem	Zero (R)	Zero (R)
Imipenem	Zero (R)	8 (R)
Meropenem	Zero (R)	Zero (R)
Cephalexine	Zero (R)	Zero (R)
Ceftizoxime	Zero (R)	Zero (R)
Ceftriaxone	Zero (R)	Zero (R)
Cefepime	12 (R)	Zero (R)
Levofloxacin	11 (R)	12 (R)
Ciprofloxacin	Zero (R)	Zero (R)
Tigecycline	10 (R)	12(R)
Colistin	12 (R)	11 (R)
Cefuroxime	Zero (R)	Zero (R)
Cefotaxime	Zero (R)	Zero (R)

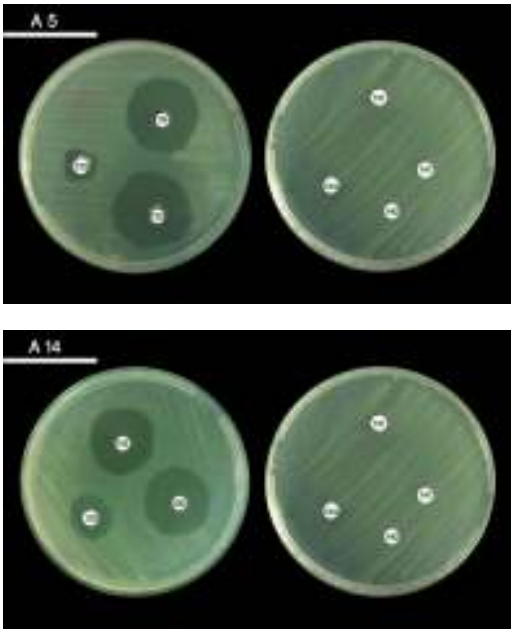

Figure (4): Antimicrobial activity of soil extracts KTD 119, KTD 120 and KTD 123 on *Acinetobacter spp.* A5 and A14 by cup-plate method

Statistical Analysis:
Statistical analysis of all tests data was reported as mean ± standard deviation and analyzed statically by using Microsoft Excel 2007. Mean ± standard

deviation for susceptible zone of inhibition for standard antimicrobial agent that were used is in this study was calculated for *Methicillin Resistant S. aureus (MRSA)* and *Acinetobacter baumannii* which equals to (18.3 ± 3.9- 27 ± 10.8) and (14.3 ±6.3- 30 ± 0) respectively.

Bacillus species KTD 119 had superior activity on MRSA and *Acinetobacter baumannii. One Streptomyces spp. (KTD 123) have moderate activity, while Bacillus spp.* have extremity activity against Acinetobacter *baumannii* (Table 5).

Table (5): Statistical analysis of antimicrobial activities by isolates on pathogenic bacteria used in this study

Test Organism	Mean ± SD						
	KTD 119	**KTD 120**	**KTD 123**	**NF 131**	**KTD 133**	**NF 140**	**NF 141**
Methicillin Resistant S. aureus (MRSA)	27 ± 10.8	18 ± 3.9	14 ± 4	18 ± 5.9	13 ± 3.5	ND	23 ± 5.7
Acinetobacter	30 ± 0	30 ± 0	14 ± 6.3	0	0	0	0

ND: Not Determined

DISCUSSION

Natural compounds obtained from soil source play important roles to discover various new drug molecules. *actinomycetes* are the most potent industrially important microorganism, which are capable of synthesizing bioactive compounds such as enzymes, hormones, vitamins and other secondary metabolites. These bioactive compounds are highly difficult to be synthesized artificially. Hence, these microbial compounds are most prominent sources for discovering and production of new drugs [16].

This study gave clear evidences that n-butanol extract from *streptomycetes* and *Bacillus* species isolated previously from Jordanian soil by Al-Shammari *et al.,* [7] showed antimicrobial activity against bacteria isolated from patients admitted at Islamic hospital in Amman-Jordan. Reports showed that most of the *actinomycetes* isolated from the soil produced antibiotics were from the genus *Streptomyces*. Not surprisingly for this finding, the Genus *Streptomyces* were found to produce more than 100,000 bioactive compounds that are of biological interest. At the same time, we should put in consideration that thousands of microbial metabolites produced from *actinomycetes* about 150-160 (0.2-0.3%) compounds were practically proved as successful lead compounds and are continuing to provide new bioactive products that lead to the discovery of many novel strains producing useful secondary metabolites [17].

Looking at the alarming situation created by the development of drug resistant pathogens, a concerted effort is being made by the scientists to search for new antimicrobial compounds that can kill or inhibit the growth of such drug resistant microbes. The choice of drugs against resistant bacteria especially (MRSA) is too hard due to their genetic alteration, enzyme variation and permeability changes [17,18].

Several studies have already been conducted on the soil *streptomycetes* of Jordan [19-23]. However, these screening studies were conducted to show the ability of the *Streptomyces* isolates to produce inhibitory affects against wild and resistant - bacteria.

In present study *Streptomyces and Bacillus* species which isolated from two locations of Jordanian soil, represented a major portion of microorganisms in soil were able to produce a wide variety of antibiotics with antibacterial activity and appeared promising.

Seven bacterial species belong to *streptomyces* and *bacillus* isolated in our previous study from soil samples taken from Jordan soil in 2011 [7] were subjected to primary screening and showed anti-bacterial activity. The bioactive compounds are extracted from natural sources through several techniques. Solvent extraction is usually employed for the extraction of secondary metabolites from the culture broth. Different Polaris of organic solvents have been utilized for the extraction of bioactive compound from *actinobacteria*. N-butanol was found to be the most suitable solvent for extraction of the culture broth of the soil isolates studied in present work [8,10,17].

Eleven clinically pathogenic bacteria species' that showed resistance to antibiotics and isolated from patients including 9 *Staphalyocoous aureus* (MRSA), obtained from various sources like urine, ear, skin, pus, diabetic foot and wounds *and 2 Acinetobacter baumanni* isolated from urine and

diabetic foot were used as target studying. They were screened for their sensitivity or resistance to several standard antibiotics according to NCCLS approved standard M100-S17.

Antibiotics used to treat serious, multiple drug resistant MRSA infections include vancomycin, as well as newer drugs such as linezolid, tigecycline, quinupristin/dalfopristin and daptomycin. Isolates with resistance to some of these drugs, including vancomycin, have been reported [24]. Ceftaroline and ceftobiprole (new fifth generation cephalosporins) are the first beta-lactam antibiotics approved in the US to treat MRSA infections (skin and soft tissue only) [25]. There has been an increase in resistance to fluoroquinolones among isolates of *S. aureus* in recent years. In this study, the highest resistance was shown by 100% for *Acinetobacter spp.* The antimicrobial activity of the n-butanol extract of streptomyces and *bacillus* against clinical isolates of resistant bacteria was determined by Agar well diffusion method.

Bacillus KTD 119 isolates gave 38 (mm) and 42 (mm) zone of inhibition on both MRSA isolates Y100 and Z100 which not obtained by other known tested antibiotics, while *Streptomycetes* soil isolates NF 141 gave 30(mm) zone of inhibition on both MRSA isolates E100 and U100, which were similar to the zone of inhibition obtained from levofloxacin and ciprofloxacin.

Streptomyces (KTD 123) and *Bacillus* species namely (KTD 119, KTD 120, NF 131) showed zone of inhibition range from 20 (mm) to 26 (mm) which was similar to those obtained by amikacin, gentamicin, cefepime and sulfamethoxazole trimethoprim. Synergism effect between *streptomyces* NF 141*, Bacillus* KTD 119 and *Bacillus* KTD 120 was seen in two MRSA isolates E100 and Y100 was showing in (Figure 3), which is good recommendation for combination of bioactive metabolites between *Streptomyces* NF 141 and *Bacillus* species (KTD 119 and KTD 120) to get synergetic effect, also we reported that *Bacillus* KTD 119 isolates give duplicate the zones of inhibition that were obtained from different tested standard antibiotics.

Statistical analysis of all tests data was reported as mean ± standard deviation and analyzed statistically by using Microsoft Excel 2007. *Bacillus* soil isolates KTD 119 showed a maximum activity against (MRSA) with mean diameter of zone inhibition 27 (mm) ± 10 and *Acinetobacter spp.* with mean diameter zone of inhibition of (24) mm ± 5.5 and (30) mm ± 0 respectively. Other *Bacillus* isolate KTD 120 showed a maximum activity against *Acinetobacter spp.* with mean diameter of zone inhibition 30 (mm) ± 0

Selvameenal *et al.* reported that the Actinomycete strain, *Streptomyces hygroscopicus* subsp. ossamyceticus (strain D10) showed activity on (MRSA), several similar studies reported that the bioactive extracts supernatant from soil bacteria was found to inhibit the growth of multi-antibiotic *S. aureus* and *Acinetobacter baumanni* [26,27].

Streptomycetes soil isolates NF 141 showed a maximum activity against (MRSA) with mean zone of 23 (mm) ± 5.7. None of *Bacillus* species (NF 131 and KTD 133) isolates or two *Streptomycetes species* (NF 140 and NF 141) isolates have the ability to produce antimicrobial activity against *Acinetobacter spp.*These results for *Bacillus* species strain KTD 119 was superior among other bioactive substances extracted from *Bacillus* species followed by *Streptomyces* strain NF141. Other strains (*Bacillus* KTD 120, *Streptomyces* KTD123, *Bacillus* KTD133 and *Bacillus* NF 133) shows variable activity that inhibit (MRSA). Two (MRSA) strains (Y 100 and E 100) were still resistance to *Bacillus* KTD 133. This promising finding was one goal of our study to look for new product that inhibits MRSA.

Bacillus isolates (KTD 119, KTD 120 and KTD 133) and *streptomyces* (KTD 123) isolated from King Tala Dam in Jordan shows activities against (MRSA). Antibiotics developed from marine microbes are particularly important because they have high potency when compared with terrestrial counterparts

The study also is substantiated by reports by Ibrahim [(28)] who isolated and identified the antibacterial activity of the *Actinomycetes* strains from four different terrestrial soil locations of King Saud University, Riyadh, Kingdom of Saudi Arabia. Antibacterial activity of the isolates was antagonistic against Gram-positive *Staphylococcus aureus* and Gram-negative bacteria *Escherichia coli.*

From the results mentioned in this study, it is possible to conclude that all the soil isolates under investigation have significant antimicrobial activity. The isolates belong to *Streptomyces* group and *Bacillus* group confirming similar earlier reports that most of the *Actinomycetes* and *Bacillus* isolated from the soil produce antibiotics*,* also this study confirm the occurrence of bacteria resistant to all most the common and widely used antibiotics in Jordan, and the picture were cleared by isolation of (MRSA) and ESBL bacteria from patients in Islamic Hospital in Amman. The ability of n-butanol extract product from our isolates to inhibit

the growth of these resistant bacteria to known antibiotics represents very good achievements.

REFERENCES

1. O'Neill J. (2014) Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations. https://www.who.int/news/item/29-04-2019-new-report-calls-for-urgent-action-to-avert-antimicrobial-resistance-crisis.

2. World Health Organization. (2017). Prioritization of pathogens to guide discovery, research and development of new antibiotics for drug-resistant bacterial infections, including tuberculosis WHO/EMP/IAU/2017.12

3. Gordon MC. and Newman JD. (2005) Biodiversity: a continuing source of novel drug leads. *Pure Appl Chem.* 77(1):7–24.

4. Al-Shammari AJN.; Mahmoud S. and Masalha I. (2013). Prevalence of resistant bacteria among patient in an Islamic hospital in Jordan. *Int. J. Sci. Technol.* 8 (4): 96-105

5. Maragakis L. and Perl T. (2008). *Acinetobacter baumannii:* Epidemiology, Antimicrobial Resistance, and Treatment Options. *Clin. Inf. Dis.* 46 (8):1254–1263.

6. Isnansetyo A. and Kamei Y. (2003). MC21-A, a Bactericidal Antibiotic Produced by a New Marine Bacterium, *Pseudoalteromonas phenolica* sp. nov. O-BC30T, against Methicillin-Resistant *Staphylococcus aureus.* Antimi. *Agents and Chemoth.* 47 (2): 480–488.

7. Al-Shammari AJN.; Sheerdia Z. and Kasam T. (2015). Isolation and characterization of antibiotics produced from Jordanian soil microorganisms. *Int. J. Sci. Technol.* 10(3): 62-69.

8. El-Najjar MY.; Hassan MA. and Said WY. (2001). Isolation and characterization of an antimicrobial substance produced by *Streptomyces violatus*. *Egypt. J. Biol.* 3:11-21

9. Aysel U. and Nurettin S. (2003). Investigation of the antimicrobial activity of some Streptomyces isolates. *Turk J. Biol.* 27:79-84.

10. Ebelle Etame RM.; Mouokeu RS.; Poundeu FSM; Voukeng IK.; Cidjeu CLP. *et al.* (2019). Effect of fractioning on antibacterial activity of n-butanol fraction from Enantia chlorantha stem bark methanol extract. *BMC Complem. Altern. Med.* 19(1): 56-61.

11. Nabi U. and Felsenstin J. (2006). Thin-layer chromatographic separation of penicillins on stannic arsenate- cellulose layer. *Acta chromatographica* 16: 164-172

12. Dobercky J.; Vazquez E.A. and Amper R. (1968). Antibiotics chromatography. SAFYBI 8:204-208.

13. CLSI. (2015); Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically approved standard. 10th ed. 35(2): 1–15.

14. Rose B. and Miller R. (1939). Studies with the agar cup-plate method. A standardized agar cup-plate technique. *J. Bact*. 38:525-537.

15. National Committee for Laboratory Standards. (2007). Performance standards for antimicrobial susceptibility tests. Seventeenth Informational supplement. NCCLS. Approved standard M100-S17.

16. Gebhardt O.; Bharti SK.; Kaur HD. (2007). Novel cyclic decapeptide antibiotics produced by Streptomyces sp. Tii-6071, Taxonomy, fermentation, isolation and biological activities. *J. Antibiot.* 54(5):428-433.

17. Firn RD. and Jones CG. (2000). The evolution of secondary metabolism – a unifying model. *Mol. Microbiol.* 37**:**989–994.

18. Emily A.; Morell B. and Balkin B. (2010). Methicillin-resistant Staphylococcus Aureus: A Pervasive Pathogen Highlights the need for new antimicrobial development. *Yale. J. Biol. Med.* 83(4):223-233.

19. Abu- Saud M. and Saadon I. (1991). Streptomyces flora of some Jordan Valley soils: characteristics and seasonal distribution. *Dirasat* 18B: 66-75.

20. Al-Shammari AJN.; Qato MK.; Semreen MH. (2006). Isolation of Halophilic bacteria from the Dead Sea produced antibiotic-Like activities. Proceeding of the" 1st International Egyptian-Jordanian Conference on Biotechnology and Sustainable Development: Current Status and Future Scenarios". Pp.: 233-236.

21. Saadoun I. and Gharaibeh R. (2003). The *Streptomyces* flora of Badia region of Jordan and its potential as a source of antibiotics active against antibiotic-resistant bacteria. *J Arid. Environ.* 53:365–371.

22. El-Banna N.; El-Banna H. and Qaddoumi S. (2018). Isolation and characterization of actinomycetes with antimicrobial activity from the soil and the effect of the environmental factors on their antimicrobial activity. *Afr. J. Microbiol. Res.* 12(35): 848-856

23. Massadeh M. and Mahmoud SM. (2019). Antibacterial Activities of Soil Bacteria Isolated from Hashemite University Area in Jordan. *Jordan J. Biol .Sci.* 12 (4):503-511.

24. Hiramatsu K.; Hanaki H.; Ino T.; Yabuta K.; Oguri T. and Tenover FC. (1997). Vancomycin-Intermediate Staphylococcus aureus in Korea. *J. Antimicrob. Chemoth.* 40 (1) 135-136.

25. Tenover F.; Weigel L., Appelbaum P.; McDougal L. *et al.* (2004). Vancomycin-Resistant Staphylococcus aureus Isolate from a Patient in Pennsylvania. Antimicrob. *Agents Chemother.* 48; 275-280.

26. Selvameenal L.; Radhakrishnan M. and Balagurunathan R. (2009). Antibiotic Pigment from Desert Soil Actinomycetes; Biological Activity, Purification and Chemical Screening. *Indian. J. Pharm. Sci.* 71(5): 499–504.

27. Al-Shammari AJ; Al-Douri E. and Mustafa M. (2005). Characterization of Methicillin Resistant Staphylococcus aureus MRSA isolated from Baghdad Hospitals by protein and plasmid profile proc. of 3rd National conference for biotechnology. Sahba , Libya

28. Ibrahim ME. (2019). Prevalence of *Acinetobacter baumannii* in Saudi Arabia: risk factors, antimicrobial resistance patterns and mechanisms of carbapenem resistance. *Ann. Clin. Microbiol. Antimicrob.* 18:1-10.