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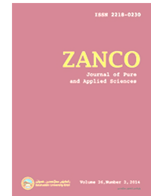

Morphological and Molecular Identification of *Trichophyton mentagrophytes* Isolated from Dermatophytes Patients in Garmian Area

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ABSTRACT

Conventional and molecular diagnosis considered as a complementary approach for making a final decision about causative agent of all microbial population, the present study was conducted in Kalar General Hospital, Dermatology unit and research laboratory center in Garmian University. Out of thirty clinically collected specimens only five samples displayed positive dermatophytic characters against microscopic and macroscopic as well as biochemical tests, such appearances of isolated colonies as white and brown color for both surface and reverse respectively, as well as, the colonies showed with a cottony texture which changed to powdery-granular colonies after two weeks incubation. Microscopic examination appeared numerous single-celled, spherical shaped microconidia were seen as clustered on both sides of hyphae, furthermore, multiseptate cigar shaped macroconidia and spiral hyphae were seen during the formation of granular colonies, biochemically analysis showed positive for urease , in addition, hair perforation testes were revealed positive results for isolates. Molecular identification carried out via conventional PCR protocol by using the primers set ITS1 and ITS4 which result in 700bp in agarose gel electrophoresis for all isolates. PCR-RFLP carried out by means of using *Bst*N1 digestion enzyme revealed four separated pattern bands 250,180,150 and 120bp. Sequencing of the ITS region in one of the isolated species revealed that similarity about 86% with *Trichophyton mentagrophytes* ATCC11481

1. INTRODUCTION

Skin infection by dermatophytes constitutes one of the health problems in the world (Kanbe *et al*., 2003). *Trichophyton mentagrophytes* is the most etiological agents of dermatophytosis in human and animal. In human *T. mentagrophytes* responsible for tinea pedis, tinea corporis, tinea unguium and tinea capitis (Symoens *et al*., 2011). *T. mentagrophytes* have the ability to invade the keratinized layer of the skin and causes hair, nail and skin infection which known as dermatophytosis

(Kanbe *et al*., 2003). Identification of dermatophytes performed by the combination of microscopic examination, in *vitro* cultural characteristic and other biochemical tests (de Hoog *et al*., 2000). Sometimes, identification of dermatophytes is complicated due to the similarities in the morphological characters between dermatophyte species which required a high level of scientific laboratory training (Nenoff *et al*., 2013). Molecular approaches have been used for the identification of dermatophytes at the level of species and strain (Gräser *et al*., 2008). The present study was aimed to investigate the conventional and molecular diagnosis of the causative agents of dermatophytes, as well as, characterizing their morphological and molecular analysis of genes and species.

2. MATERIALS AND METHODS

2.1 Samples Isolation

Thirty clinical specimens from infected hair, nail and frayed skin collected from suspected patients with dermatophytosis such (tinea pedis, tinea cruris, and tinea corporis) under the supervision of specialized dermatologist in the dermatology department of the General Hospital in Kalar district /Sulaimania Province. Specimens were collected from November to the end of December 2016. The specimens collected in sterile closed tubes and transferred to the research laboratory center of Biology Department at College of the Education \ University of Garmian for further mycological examination.

2.2 Phenotypic Identification

All isolates samples undergo two common tests, first as direct microscopic examination and the second through cultivating them on potato dextrose agar (PDA) and Sabouraud's dextrose agar (SDA) (at 25˚C for 21 days and examined at intervals times each three days) to study the macroscopic examination for characterizing the surface and reverse color formation and pigmentation occurrence of the fungal colonies. Direct microscopic examination carried out by 10% KOH to describe the filamentous hyphae and arthroconidia states of the dermatophytes, as well as, Direct mount examination was carried out by Lactophenol Cotton Blue (LPCB) to analyze the fungal species, in addition to microscopic and macroscopic examination, biochemical tests were achieved by hair perforation test, growth on rice grain and urease test to determine extra characterization of the isolates (Forobes *et al.,* 1998, Nenoff *et al.,* 2007 and Kanbe, 2008).

2.3 Molecular Identification

The genomic DNA was extracted from the fungal colonies by using OMEGA Fungal DNA Mini Kit/USA based on the manufacturer’s instructions. Molecular identification of the pathogenic fungi was carried out by species-specific PCR which

conducted by amplification of the internal transcript spacer (ITS) gene of the rDNA in dermatophyte species and amplified by using one pair of primers (Table 1) symbolized ITS1 as forward primer and ITS4 as a reverse primer (built-up in University of Koea\ KRG) (Refai *et al*., 2013). PCR was performed in 25µL of PCR reaction mixture containing: 12.5 µL of master mix (GeNet Bio\Korea), 2µL of DNA template, 9.5µL of deionized water and 0.5µL from each primer (forward primer ITS1 and reverse primer ITS 4).

The PCR mixtures were spun down shortly for 5-10 seconds then placed in thermal cycler (TCY, Crealcon, NL) and subjected to the following cycling conditions according to Ghojoghi, *et al*., (2015): initial denaturation at 95°C for 1minute, followed by 35 cycle of denaturation at 95°Cfor 30 seconds, annealing at 55°C for 1minute and extension at 72°C for 2minutes and a final extension step at 72°C for 5minutes.The amplified DNA fragments were visualized in 1.5% agarose gel electrophoresis containing ethidium bromide at 90 volts for 60 minutes at room temperature. Amplicon size determined by comparison with 100bp DNA ladder (GeNet Bio\Korea).

The PCR-RFLP analysis was conducted by using the *Bst*N1 restriction enzyme (BioLab, UK) according to the protocol provided by the company. PCR product was sequenced in Macrogene lab\ South Korea as Standard Sequencing Service.

Table 1- Primer sequences, product size and annealing temperature

Primers	Primer sequence(5′-3′)	Product size(bp)	Ann. temp.
Forward primer ITS1	F (5- TCC GTA GGT GAA CCT GCG C-3)	700	55°C
Reverse primer ITS4	R (5- TCC TCC GCT TAT TGA TAT GC-3)	700	55°C

2.4 Restriction Fragment Length Polymorphism (PCR-RFLP) Analysis

In order to the identification of dermatophytes at species level by species-specific PCR, all PCR products were subjected to digestion with the *Bst*N1 restriction enzyme (BioLab, UK) according to the protocol provided by the manufacture. The mixture contained 10µL of PCR product, 1.5µL of 10 X NEB buffer and 0.5µL of the *Bst*N1 enzyme, which recognizes the sequence 5' CC (T/A) GG 3' .Subsequently, reactions were incubated at 60°C in a water bath for one hour. 10 µL of each PCR digested products and 5 µL of DNA ladder were electrophoresed on 3% agarose gel at 90 volts for 90 minutes at the room. The gel was observed under a UV transilluminator and the DNA bands were visualized and photographed with a digital camera, then compared the electrophoretic RFLP pattern with those profiles (Jackson-Fisher *et al.,* 1999; Rezaei-Matehkolaei *et al.,* 2012; Elavarashi *et al.,* 2013).

3. RESULTS AND DISCUSSION

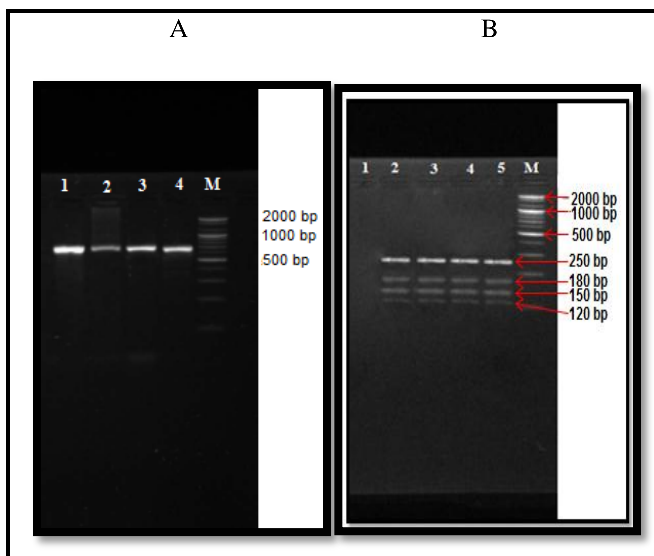
The collected samples from (scraping skin, clipping nail and hair fragments) were treated with 10% KOH test and conventional laboratory identification which include macroscopic, microscopic and biochemical tests. Out of thirty collected specimens, only five of them (16.67%) displayed positive results against 10% KOH test, while the rest of all other isolates showed negative results.

Microscopically all five isolates were showed numerous single-celled and globose shaped microconidia were seen as a clustered on both sides of the hyphae, furthermore, Multiseptate cigar shaped macroconidia were seen during the formation of granular colonies (Fig. 1), as well as spiral hyphae were seen during the microscopic slide preparation under 40X. Singh and Beena, (2003) also conducting positive and negative results by direct microscopically examination

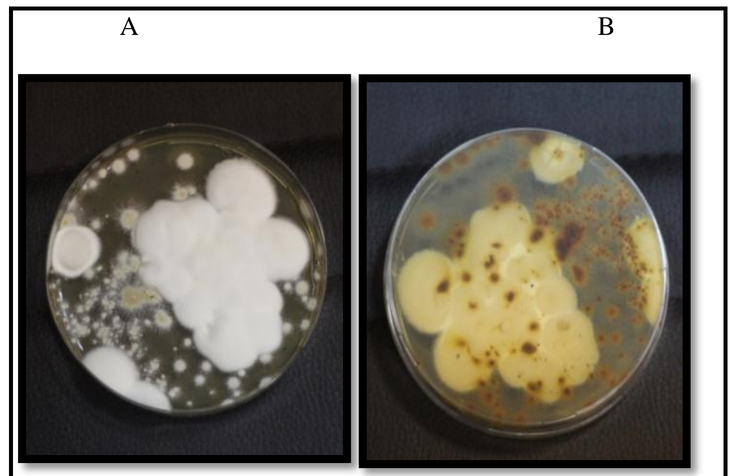
Macroscopically analysis revealed that the growing colonies on PDA and SDA at 25 °C for the first week of incubation appeared as a flat with cottony texture which then produced powdery to granular colonies possessing radial margin, while in the study of Poluri *et al.,* (2015) revealed that dermatophyte test medium (DTM) is better than SDA for screening and identification of dermatophyte species, on the other hands, the colonies pigmentation color appeared with white color in the surface side, while in the reverse side pigmentation revealed as brown color (Fig. 2).The biochemical analysis was done for the obtained colonies exhibit as positive results for urease test, as well as, also same results for hair perforation test were achieved, in addition, the biochemical identification system also can be useful to identified some of the fungus strains depending on their phenotypically and their biochemical characteristics as discussed by Maikhan and Mohammed-Amin, (2017) . The obtained results was in agreement with that discussed by Surendran *et al.,* (2014), that the culturing may identify the fungus species till the species, moreover it will require other tests in order to determine their genus.

Molecular identification was by species-specific PCR carried out by amplification of the ITS region on the ribosomal DNA by using the set of universal primers ITS1 and ITS4. Amplification of the ITS region resulted in PCR product about 700bp when electrophoresed on 1.5 % agarose gel, while the digestion of the amplified ITS product by *Bst*N1 restriction enzyme during the performing of PCR-RFLP revealed four patterns 250,180,150 and 120 bp through the electrophoresis in 3% agarose gel (Fig. 3). The profiles of electrophoretic analysis of patterns were obtained from ITS- RFLP in all clinical isolates showed similarity with those of Elavarashi *et al*., (2013) and Ahmadi *et al*., (2015); where they identified dermatophytes by PCR-RFLP and described the same patterns of ITS-RFLP. For species identification, ITS

sequence of clinical isolates aligned with the reference sequence recorded in the database by using BLAST sequence analysis tool ″http:www.ncbi.nlm.gov\BLAST″ from the National Center for Biotechnology Information. According to the sequence of the ITS region in rDNA of four clinical isolates were identified as *Trichophyton mentagrophytes* revealed that only one isolate showed similarity about 86% with *Trichophyton mentagrophytes* ATCC11481 (Fig. 4) and *Arthroderma simii* strain: CBS150.66 (Figure 5), and also showed 84% identity with a number of species such as *Trichophyton quinckeanum strain* ATCC 11480 and *Trichophyton schoenleinii strain* ATCC 22775. While the sequence of the ITS region in the other isolates of revealed uncultured fungus during the search process in NCBI database information. Relied on Yu *et al*., (2004), the ITS regions have interspecies



polymorphism and few variance at the level of intra species. Consequently, the sequencing of the ITS region in the rDNA in dermatophytes proved to be useful for the identification and resolving the phylogenetic relationship between dermatophyte fungal species (Dhieb *et al*., 2014).The sequencing method of rDNA



ITS fragments presently considered the gold standard for identification of strains in the *T. mentagrophytes* complex, (Li *et al.,* 2008)

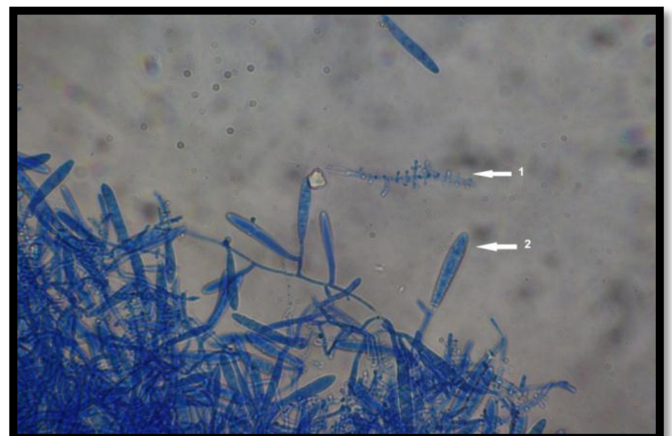


FIGURE (2): The colonies pigmentation color after three weeks of incubation at 25 °C on SDA, (panel A showing surface side pigmentation, panel B displayed reverse side pigmentation).

FIGURE (3): A. Agarose gel electrophoresis of PCR products of DNA amplification. M: 100 bp molecular marker. Lanes1-4: ITS region of *Trichophyton mentagrophytes* (700bp), B. Agarose gel electrophoretic profile of ITS-RFLP with *Bst*NI of local isolate of *Trichophyton mentagrophytes*. M: 100 bp molecular marker. Lane 1: Negative control. Lanes 2-5: restriction digested of DNA (250bp, 180bp, 150bp, and 120bp)

Score	**Expect**	**Identities**	**Gaps**
383 bits(207)	5e-102	310/360(86%)	6/360(1%)

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Query  86   ACCTGCACC-TTCTTGT-TTCCTTACTCGGATGCCTCCGCCGGACGCCCTCTCTC-CTTG  142
            ||| || || ||||||| | |||||||||| |||||| || || ||| ||||||| |  |
Sbjct  50   ACC-GCCCCATTCTTGTCTACCTTACTCGGTTGCCTCGGCGGGCCGCGCTCTCTCTCAGG  108

Query  143  AGATTCGTTCGGTGAGTCTCTCTTTTTTGGCTCCACGTTGGACTTCGCCCGCCCGACGAC  202
            |||  ||||||| ||| ||||||||  |||||| ||| |||||  |||||||| || |||
Sbjct  109  AGAGCCGTTCGGCGAGCCTCTCTTTAGTGGCTCAACGCTGGACCGCGCCCGCCGGAGGAC  168

Query  203  AGACGCCTTGTATATTCCTTCATATCACCTGTCAATCTGAGCGTTATCTAGCAAAAATCA  262
            ||||||     | |||| |||| |  | |||||| ||||||||||| | |||||||||||
Sbjct  169  AGACGC--AAAAAATTCTTTCAGAAGAGCTGTCAGTCTGAGCGTTAGCAAGCAAAAATCA  226

Query  263  GTTAAAACTTTCTACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCTTCGAAATG  322
            |||||||||||| ||||||||||||||||||||||||||||||||||||||  |||||||
Sbjct  227  GTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATG  286

Query  323  CAATAAGTAATGTGAATTGCACAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCG  382
            | ||||||||||||||||||| ||||| ||||||||||||||||||||||||||||||||
Sbjct  287  CGATAAGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTTTGAACGCACATTGCG  346

Query  383  CCCCCTGGTATTCCGGGGGGCATGCCTGTTCGCGCGTCGTTTCACACCCTCAAGCCGGGC  442
            |||||||||||||||||||||||||||||||| ||||| |||||  |||||||||| |||
Sbjct  347  CCCCCTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCAGCCCCTCAAGCCCGGC  406
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FIGURE (4): Partial Sequence of the Internal Transcript Spacer (ITS) in locally isolate of *Trichophyton mentagrophytes* with *Trichophyton mentagrophytes* ATCC11481

Score	Expect	Identities	Gaps	Strand
383 bits(207)	5e-102	309/359(86%)	4/359(1%)	Plus/Plus

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Query  86   ACCTGCACC-TTCTTGT-TTCCTTACTCGGATGCCTCCGCCGGACGCCCTCTCTCCTTGA  143
            ||| || || ||||||| | |||||||||| |||||| || || ||| ||||| ||  ||
Sbjct  69   ACC-GCCCCATTCTTGTCTACCTTACTCGGTTGCCTCGGCGGGCCGCGCTCTC-CCAGGA  126

Query  144  GATTCGTTCGGTGAGTCTCTCTTTTTTGGCTCCACGTTGGACTTCGCCCGCCCGACGACA  203
            ||  ||||||| ||| ||||||||  |||||| ||| |||||  |||||||| || ||||
Sbjct  127  GAGCCGTTCGGCGAGCCTCTCTTTAGTGGCTCAACGCTGGACCGCGCCCGCCGGAGGACA  186

Query  204  GACGCCTTGTATATTCCTTCATATCACCTGTCAATCTGAGCGTTATCTAGCAAAAATCAG  263
            |||||     | |||| |||| |  | |||||| ||||||||||| | ||||||||||||
Sbjct  187  GACGCAAAAAAAATTCTTTCAGAAGAGCTGTCAGTCTGAGCGTTAGCAAGCAAAAATCAG  246

Query  264  TTAAAACTTTCTACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCTTCGAAATGC  323
            ||||||||||| ||||||||||||||||||||||||||||||||||||||  ||||||||
Sbjct  247  TTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGC  306

Query  324  AATAAGTAATGTGAATTGCACAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGC  383
             ||||||||||||||||||| ||||| |||||||||||||||||||||||||||||||||
Sbjct  307  GATAAGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTTTGAACGCACATTGCGC  366

Query  384  CCCCTGGTATTCCGGGGGGCATGCCTGTTCGCGCGTCGTTTCACACCCTCAAGCCGGGC  442
            ||||||||||||||||||||||||||||||| ||||| |||||  |||||||||| |||
Sbjct  367  CCCCTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTTCAGCCCCTCAAGCCCGGC  425
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FIGURE (5): Partial Sequence of the Internal Transcript Spacer (ITS) in locally isolate of *Trichophyton mentagrophytes* with *Arthroderma simii*

4. CONCLUSIONS

The following can be drawn out of this paper:

a. Conventional and molecular diagnosis considered as a complementary approach for making a final decision about causative agent of all microbial population
b. Microscopic examination and biochemical analysis together facilitated primary identification of the obtained dermatophytes genus
c. Molecular identification with exact set of primers ITS1 and ITS4, as well as by using *Bst*N1 digestion enzyme revealed four separated pattern bands 250,180,150 and 120bp.
d. The ITS region in sequencing revealed that similarity about 86% with *Trichophyton mentagrophytes* ATCC11481.

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Isolation and Identification of *Burkholderia mallei* and its Susceptibility to *Anethum graveolens* Extracts

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A B S T R A C T

In the present study, 13 (4.06%) isolates of *Burkholderia mallei* were obtained from hands of 320 workers in veterinary laboratories in Erbil Province- Iraq. All isolates identified depending on conventional assays, VITEK 2 compact system, and further confirmed by PCR using 23 rDNA gen. The results of PCR product showed that the expected size (526bp) was obtained for all local isolates and for *B. mallei* ATCC 15310. Also the effect of 10 antimicrobials against isolated bacteria were studied and the results showed that isolated *B. mallei* were absolutely resistant to norfloxacin, and 100% sensitive to imipenem and ceftazidime. The antibacterial activity of aqueous and alcoholic extracts of *Anethum graveolens* against *B. mallei* was studied. The results of MIC by using broth dilution method revealed that the alcohol and aqueous extracts exhibited inhibition of *B. mallei* growth and the MIC was 800, 1000 µg/ml, respectively. On molecular study, the SubMIC (750, 950 µg/ml) for both extracts against plasmid DNA profile of most resistant isolate of *B. mallei* was studied. The results showed three plasmid DNA band exist in local isolate of *B. mallei* and stander strain of *B. mallei* ATCC 15310 before treating with *A. graveolens* extracts. While it was decreased from three to two in the local isolate and the standard strain ATCC 15310 when treated with *A. graveolens* extracts. The inhibitory effect of the above mentioned extracts against total protein was studied by SDS-PAGE binding pattern. The results showed that there were differences in the protein banding pattern among the tested isolates and induction of some new protein bands when treated with plant extracts.

1. INTRODUCTION

B.mallei is a zoonotic Gram-negative intracellular bacterium. It is an etiologic agent of glanders, a disease primarily of equids, and it is also communicable to man with fatal consequences (Whitlock *et al.* 2007). This bacterium was one of the agents used in biologic warfare during the 20thcentury (Rotz *et al*., 2002), and the disease still occurs in Asia, Africa and South America and in the last decade, the disease outbreaks were reported in different areas including; Brazil, Eretria, Ethiopia, Iran, Iraq, India, Pakistan and United Arab Emirates (OIE, 2008; Dvorak & Spickler, 2008; Naureen *et al*., 2007). The direct contact of people with glanderous animals such as veterinarians, farmers or laboratory workers cause severe human infections and it is transmit through the contact of infectious exudates with cuts and abrasions or with mucosal membranes (Horn, 2003). *B. mallei* are highly infectious organisms by aerosol and can cause different severe diseases such as septicemia, pulmonary or chronic infections of the muscle, spleen and liver (Mandell and Dolin , 2006).

Treatment of bacterial diseases is ideally guided by the results of in vitro antibiotic susceptibility test. According to many previous studies, the *B. mallei* intrinsically resistant to a wide range of antimicrobial agents including b-lactam antibiotics, aminoglycosides and macrolides….etc. (Heine *et al.* 2001; Dance *et al*. 1989). In recent years, the increasing prevalence of multidrug resistant bacteria and appearance of strains with reduced susceptibility to antibiotics raises an urgent need to search for discovering a new antimicrobial compounds (Mustafa, 2007).

Medicinal plants are heavily and worldwide used in folk medicine and we shall not forget that most drugs of the past were substances with a particular therapeutic action extracted from different plants. The plant compounds that can either inhibit the growth or kill the bacteria and have no or least toxicity to host cells are considered candidates for developing new antimicrobial drugs. Iraq has a rich flora and a wide knowledge of its indigenous medicinal plants. Also it was found that food and their individual constituents perform similar fashion to modern drug and may be better without the dreaded side effect (Arora and Kaur, 2007; Mustafa *et al.,* 2012). *A. graveolens* (Dill) is a plant belong to family Umbelliferae which is instantly recognized by its distinctive minty smell. *A. graveolens* has an Asia west and Mediterranean distribution and it is widespread in Euroasia (Bailer *et al*. 2001). The antibacterial effect of dill is a sparse looking plant with feathery leaves and tiny yellow flowers. Some pharmacological effects have been reported, such as antimicrobial and gastrointestinal ailments (Delaquis *et al.*, 2002; Duke, 2001).

The objectives of this study were isolation and identification of *B. mallei* in Erbil province

which entails a particular risk of infection for laboratory personnel by using VITEK 2 Compact system and PCR technique. The antibacterial activity of dill extracts was studied against resistant isolates by using different methods.

2. MATERIALS AND METHODS

2.1 *B. mallei* isolates

Thirteen isolates of *B. mallei* were obtained from hands of 320 workers in veterinary laboratories in Erbil Province, Iraq. Culture and identification of *B. mallei* was carried out following procedures; briefly, swab samples were cultured on brain heart infusion agar (BHI agar, Oxoid, UK) supplemented with sheep blood. The bacterial colonies were screened for Oxidase, catalase, indole and colistin resistance. The all isolates confirmed by VITEK 2 Compact system as *B. mallei*. Also these isolates were further confirmed by PCR.

2.2 Detection of *B. mallei* local isolates by using PCR

All molecular techniques used in this study depended on Sambrook and Russell (2001). For DNA extraction, G-spin™ Genomic DNA extraction kit was used. The kit utilizes silica-based membrane technology in the form of a convenient spin column. The genomic DNA purified and DNA was extracted from isolates and used for detection by PCR technique as mentioned by (iNTRON Biotechnology, South Korea) and the purified DNA immediately stored at -20 °C. For 23S rDNA gene the oligonucleotide primer (5′-CAC CGA AAC TAG CA-3′ with product size 526bp (Bauernfeind *et al*., 1998)) was purchased from Fermentas, Germany.Amplification reaction was performed in a 50µl final volume with 1 U of *Taq* polymerase (Fermentas, Germany), 5 µl of the reaction buffer was diluted 1:10, a 10 µM concentration of each deoxynucleotide triphosphate, and a 50 pM concentration of each oligonucleotide primer. Moreover, approximately 50 to 100 ng of DNA template was used in amplification. The PCR initial denaturation step of 5 min at 95°C followed by 25 amplification cycles of 30sec at 95°C, 30 sec at the primer-specific annealing temperature 47°C and 45 sec at 72°C. The samples were then incubated at 72°C for another 7 min and cooled to 4°C. Double-distilled, sterile water instead of template DNA was used as the negative control to exclude amplicon contamination. The amplification products were checked by agarose gel electrophoresis and subsequently purified by using the PCR purification kit (Qiagen, Germany) to desalt and remove excess primers. Finally the PCR products were analyzed by gel electrophoresis using 1% agarose gel. PCR product samples were mixed with 6 x loading dye. Bands were visualized and photographed digitally using ChemiDoc-it^2 Imager Transilluminator UV (U.S.A.). The results were compared with *B. mallei* ATCC 15310.

2.3 Preparation of bacterial inoculum

The isolated bacteria were grown in Mueller-Hinton broth (MHB) for 24 h at 37 °C. The cultures were adjusted to achieve $1x10^6$ colony forming units (CFU/ml).

2.4 Antimicrobial resistant test

In vitro antimicrobial susceptibility to 10 antimicrobials was determined by Kirby Bauers disk diffusion method as described by (CLSI 2011). The antimicrobials were amoxicillin (25µg), ampicillin (10µg), chloramphenicol (30µg), gentamycin (30µg), co-amoxiclav (30µg), ciprofloxacin (10µg), imipenem (10µg), ceftazidime (30µg), norfloxacin (10µg), and levofloxacin (5µg).

2.5 Collection and preparation of plant extracts

Leaves of *A. graveolens* were obtained from local vegetable market in Erbil city / Iraq. They were carefully washed, oven-dried for 1 h at 160°C and put in the shade in an aerated place till complete drying, then were ground into a fine powder with a blender machine. The prepared powder was soaked in each of water and ethanol solvents (plant material to solvent ratio was 1:10, w/v) and extracted for 24 h at room temperature with shaking at 150 rpm. The extracts were filtered using Whatman filter paper (No.1). The filtered was collected and evaporated under vacuum using the BUCHI Switzerland rotary evaporator to obtain concentrated, powdered extracts. The extracts were then kept in sterile bottles under refrigerated conditions until further use (Saei-dehkordi *et al*., 2014).

2.5.1Antimicrobial screening tests

The antibacterial effect of *A. graveolens* extracts was studied by using broth dilution method. Different concentrations of the extracts were prepared by nutrient broth medium to give concentrations ranged from 50 to 1500 µg/ml for each aqueous and ethanol extracts separately. Single colonies of the tested *B. mallei* were used for inoculation of LB media containing the above leaves extract concentrations and were incubated at 37°C for 24h as described by Baeshin *et al*. (2005).

2.5.3 Extraction, purification of plasmids

Plasmids were extracted by alkaline lysis method with some modification. Plasmids were extracted from the bacterial strains before and after the treatment with the SubMICs concentrations of plant extracts. Isolates were inoculated in LB medium and centrifuged, pellet were re-suspended in 100µl of GTE buffer (10 ml Glucose 0.5 M, 2.5 ml Tris-base 1 M pH=8, and 2 ml EDTA 0.5 M). Two hundred microliter of lysis buffer (1 ml NaOH 1 M and 0.5 ml SDS 10%) was added and incubated in ice for 10 minutes. After that cold sodium acetate solution was added and incubated on ice for 15 minutes and centrifuged. Phenol/chloroform/isoamyle alcohol in a 1:24:25 ratio was added to

supernatant, after centrifugation isopropanol was added and again centrifuged for 15 minutes, pellet washed with cold ethanol and centrifuged finally supernatant discarded and pellet re-suspended in 50 µl of TE buffer (Tris-HCl pH= 8 10 mM, EDTA 1 mM and RNase). The isolated plasmids were then digested with the restriction enzyme Eco RI (Qiagen, Germany) using the standard protocol and digested plasmid DNA was separated in 1% agarose gel, stained with ethidium bromide and photographed digitally using ChemiDoc-it^2 Imager Transilluminator UV (U.S.A.).

2.5.4 Protein profile of *B. mallei* by using SDS-PAGE

Pure cultures of the *B. mallei* isolates were grown at 37 °C in 2 ml of the (LB) broth medium (Merck, Germany) under constant shaking at 200 rpm for 6–7 hours. For sub-culturing, 100 µL of the culture was inoculated into 10 mL of the LB medium and incubated overnight at 37 °C with constant shaking. The cells were harvested by centrifugation for 10 minutes at 4 °C and the cell pellet was washed twice using 10 mM Tris-buffer pH 7.6 buffers, re-suspended in same buffer, and 5x SDS loading dye containing beta-mercaptoethanol (Sigma, Germany). Grown cells were harvested by centrifugation at 4 °C, washed twice and re-suspended in 2.5 ml ice cold 10 mM Tris-buffer pH 7.6. In a 1.5 ml eppendorf tube, 80 µl of the cell suspension was added to 20 µl SDS loading dye (with beta-mercaptoethanol), mixed well and incubated at 98 °C for 20 minutes using an eppendorf thermostat hotplate, and stored at 4°C. To remove insoluble materials and non-lysed cells, samples were centrifuged at maximum speed for 30 minutes and 10 µl supernatants of the samples were loaded on an SDS-PAGE for protein expression. The samples were analyzed using 10% SDS-PAGE. The SDS-PAGE was performed with 5% stacking and 10% separating gel after the sample preparation was solubilized at 100 °C for 5 minutes in 0.05 M of tris-HC1 buffer (2.5% SDS, 5% 2-mercaptoethand, 25% glycerol and 0.03% bromophenol blue). Protein bands were detected after 2 h in an electrophoresis module at a constant 35 mA per gel with 120 volts using 1x electrophoresis buffer of staining 0.25% Coomassie brilliant blue R250 (Sigma, Germany) using pre-stained protein markers (ROTH, Germany). After separation, proteins were visualized and gels were photographed (BioDoc-It2 gel imaging system/USA) as described by (Sambrook and Russell, 2001).

3. RESULTS

3.1 Identification of *B. mallei*

During the present study, 13 (4.06%) of *B. mallei* were obtained from hands of 320 workers in veterinary laboratories depending on conventional assays and the irregularly stained Gram negative rods, indole negative, resistant to colistin, non- motile, triple sugar iron negative, arginine and gelatin positive

isolates were presumptively identified as those of *B. mallei* and finally all isolates confirmed by VITEK 2 compact system. On the other hand, the above results were further confirmed by PCR using 23 rDNA (Figure 1) and the results of PCR product showed that the expected size for all local isolates and *B. mallei* ATCC 15310 was (526bp).

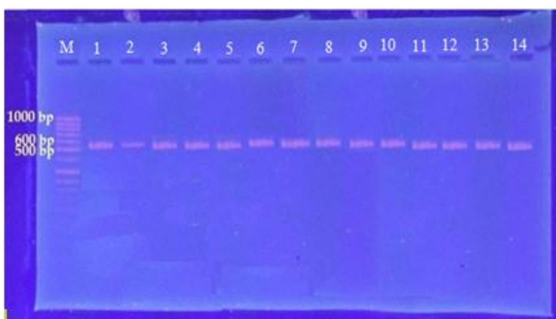

Figure 1. Agarose gel electrophoresis analysis (stained with ethidium bromide) of PCR products presenting 23S rDNA amplified from the thirteen local tested strains of *B. mallei* with expected size 526bp. Lane M : DNA Marker; 1, *B. mallei* ATCC 15310 and 2-14, local isolates of *B. mallei*.

3.2 Antimicrobial susceptibility testing

The results of studied antimicrobials against *B. mallei* are given in table (1). In the present study, all *B. mallei* isolates were absolutely (100%) resistant to norfloxacin, while 92.31%, 84.62%, 53.85%, 46.15%, 30.77% , 30.77% and 7.69% isolates showed resistance to ampicillin, amoxicillin, levofloxacin, gentamycin, chloramphenicol, ciprofloxacin, and co-amoxiclav, respectively. Whereas all obtained isolates were 100% sensitive to imipenem and ceftazidime antimicrobials.

3.3Antibacterial activity of *A. graveolens* against *B. mallei* growth

The antibacterial activity of different concentrations of aqueous and alcoholic extract of *A. graveolens* leaves against the most resistant *B. mallei* was studied by MIC by using broth dilution method. The results revealed that the alcohol and aqueous extracts of dill leaves exhibited inhibition of *B. mallei* growth and the MICs are 800, 1000 µg/ml, respectively. For the molecular study, the SubMICs (750, 950 µg/ml) for alcohol and aqueous extracts of *A. graveolens* leaves against plasmid DNA profile of most resistant isolate of *B. mallei* was studied. The results showed decreasing in the number of plasmids bands from three in untreated isolate and the standard strain ATCC 15310 to two bands in treated isolates with both extracts under study (Figure 2). However, also the inhibitory effect of the above mentioned extracts of *A. graveolens* leaves against total protein of *B. mallei* was studied by using SDS-PAGE binding pattern to know whether the both above mentioned SubMICs affect the bacterial protein or not .

Total cellular protein extracted from treated and untreated bacteria is shown in figure (3: lanes 1-4). The results of present study showed that there were differences in the protein banding pattern among the tested isolates and induction of some new protein bands due to the treatment with 750 and

950µg/ml of aqueous and alcoholic plant extracts was noticed in treated *B. mallei* as indicated by the arrows (Figures 3: lanes 1,2).

Table 1. Number and percentage of antimicrobials resistant of isolated *B. mallei*.

Antimicrobials	No. of resistant isolates n=13	Resistance (%)
Amoxicillin	11	84.62
Ampicillin	12	92.31
Ceftazidime	0	0
Chloramphenicol	4	30.77
Ciprofloxacin	4	30.77
Co-amoxiclav	1	7.69
Gentamycin	6	46.15
Imipenem	0	0
Levofloxacin	7	53.85
Norfloxacin	13	100

The analysis of these banding patterns showed that the molecular weights of bands ranged from 20 to 100 KDs

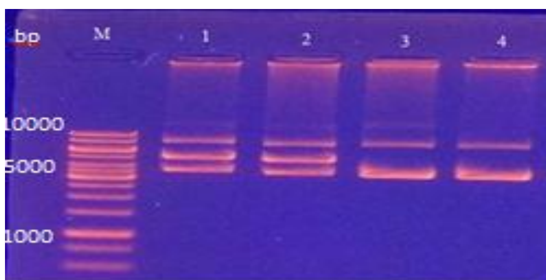

Figure 2. Plasmid DNA profile extracted from *B. mallei*. Lane M: DNA marker; 1, untreated *B. mallei* ATCC 15310; 2, untreated local of *B. mallei*; 3, after treated with 750 µg/ml of dill ethanol extract; 4, after treated with 950µg/ml of dill aqueous plant extract.

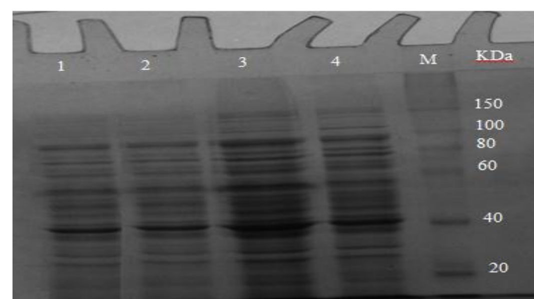

Figure 3. Protein banding pattern as revealed by SDS-PAGE of the *B. mallei* strains. Lane M: Protein marker; 1, untreated *B. mallei* ATCC 15310; 2, untreated local isolate of *B. mallei*; 3, after treated

with 750 µg/ml of dill ethanol extract; 4, after treated with 950µg/ml of dill aqueous plant extract.

4. DISCUSSIONS

Thirteen isolates of *B. mallei* was detected and identified based on colony morphology and Gram staining appearance, culture criteria, biochemical characteristics, and then confirmed by VITEK 2 Compact system. The obtained isolates from VITEK 2 were further identified by PCR based on the 23S rDNA and these results agreed with results obtained by (Bauernfeind *et al*., 1998). It is the first isolation of *B. mallei* from human sources and molecular study in Iraq. Although, in medical microbiology unequivocal identification of *B. mallei* by conventional biochemical assays is usually get on, but there is a remarkably high risk of becoming infected while working with living cultures of *B. mallei*. The infection risk could be reduced by using PCR. The identification of bacteria can then be performed with killed bacteria or the template DNA thereof. A PCR procedure for the discrimination of *B. mallei* was developed and in comparison with classic methods the procedure allows more rapid and accurate identification at reduced risk for infection of laboratory personnel.

In the present study a high proportion of resistance (100%) against norfloxacin observed that discourages the use of this antibiotic in prospective treatment trials. The obtained results might be due to the use of different, and high concentration of norfloxacin and also mutation in gyrA gene could be another inciting factor (Muhammad *et al.,* 1998; Burns, 2006). In addition to, high resistance to ampicillin, amoxicillin was found in this study might either be due to modification in PBP or the occurrence of PenA (βlactamase) gene which powers the resistance against penicillins and cephalosporins antibiotics has also been noted in *B. mallei* ATCC 23344 strain (Tribuddharat *et al*., 2003). The above mentioned results were similar to results reported by (Saqib *et al*., 2010). Nevertheless, no resistance was recorded against imipenem and ceftazidime and these antimicrobials recorded as the most effective antimicrobials against the tested bacteria. This result was similar to results have been reported by (Thibault *et al*., 2004). This study confirms the high level of antimicrobial resistance in *B. mallei* isolates and these resistance profiles appeared to be independent of the origin of isolates. It is worth to mention that the bacteria in general in Kurdistan region, Iraq are resistant to most of the antimicrobials and these emergences due to misuse of medication, use of high concentrations without physician's consultation, and lack of good quality control of medicines.

Currently, the search for new antimicrobial agents such as medicinal plants has been necessitated by the increase in antimicrobial

resistance in recent years. The natural plant products are a good source of new antimicrobials as they generally have low toxicity, have a low risk of development of resistance by pathogens, are cheap and are generally safer than synthetic medicines and have been used in traditional medicine to improve the quality of healthcare (Garvey *et al.,* 2011; Rakholiya and Chanda , 2012). One of the medicinal plants is dill plant which has a long history of cultivation, use as culinary, and medicinal herb. Our finding showed that dill leaves extracts possess a good antibacterial activities against *B. mallei* isolates by using broth dilution (MIC) assay at different concentrations and molecular studies. Phytochemical analysis of dill plant extracts illustrated the presence of alkaloid, carbohydrate, resin, terpenoids, flavonosides, saponin, steroid, tannin, and flavonoid (Dahiya and Purkayastha, 2012; Pathak *et al*., 2014). Moreover, dill oil contained various saturated and unsaturated fatty acids and this mentioned oil includes essential oil and volatile oil which can be extracted from various parts of plants including the leaves (Mahdi ,2016;) Chahal *et al*. , 2017). In addition, Orhan *et al*. (2013) found a number of phenolic acids like vanillic, caffeic, protocathechuic, pcoumaric, ferulic, chlorogenic, syringic, rosmarinic, ocoumaric and trans-cinnamic acid in ethanol extract of dill leave. The above mentioned results confirmed by reducing number of plasmid DNA bands from three bands in untreated *B. mallei* isolates to two bands when treated with both Sub MICs of both plant extracts. On the other hand, the results of SDS-PAGE protein profile of the SubMICs concentrations showed the induction of the expression of new proteins which was observed by appearance of new bands (Figure 3) and these results disagree with Mustafa and AL-Rawi (2017) that found decreasing of the protein bands of the *S. paucimobilis* when treated with *Prosopis farcta* pods extracts. These results may be due to the treatment that could be explained if we consider the ability of *A. graveolens* leaves extracts to apply a stress on the treated *B. mallei* and under this stress, treated isolates which could probably respond with increase of the expression level of some proteins and induction of others. Furthermore, the dill contents could inactivate the proteins, decreasing the membrane permeability as the consequences of cytoplasmic membrane damages and eventually causing the cellular death, our results were similar to results obtained by (El-Tarras *et al*., 2013).

5. CONCLUSIONS

In a conclusion, molecular identification of *B. mallei* is a good alternative method comparing with conventional method. According to the present study the leaves extract of the *A. graveolens* proved to have antimicrobial activities against *B. mallei.* This finding indicates the possibility of using the *A. graveolens* leave extract as a source of

antibacterial compounds for treatment of infections caused by bacterial pathogens.

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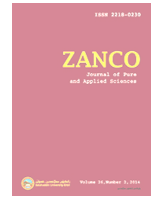

Study of some physical wool characterization in Arabi ewes in Erbil Governorate

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A B S T R A C T

This study was carried out at Private flock in Murtkah District - Erbil Governorate. Samples of wool were taken from mid-side of 72 Arabi ewes during May, 2017. to study the effect of ewes age and the sex of the lambs on the physical wool characteristics The overall means of raw fleece weight, clean fleece, fiber diameter, crimps, staple length and fiber length were 1653.75 g, 79.06%, 28.24 micron, 1.33 cm, 9.36 cm and 16.03 cm respectively. The percentages of the fibers of each of Fine, Coarse and Kemp were 52.42, 38.89 and 8.68% respectively. and S/P ratio 1.27. Age of ewe effects appeared to be significant on raw fleece weight and coarse fiber ($p \leq 0.05$), also on fine fiber and (S/P) ratio ($p \leq 0.01$) only, while the effects of sex of lamb were non-significant on all physical wool properties except the percentage of kemp fiber ($p \leq 0.05$). Although the maximum R-square increased very little gradually in the equation to predict fleece weight depending on body weight and dimensions, the simplest equation includes body weight could be considered the most significant ($P<0.01$) and more reliable than others to predict fleece weight (The equation is: fleece weight = 353.41 + 31.84 (body weight). The multiple regression equations were calculated to predict clean wool percentage, staple length, fiber length, crimps, fine fiber percentage, coarse fiber percentage, and S/P ratio from fleece weight, body weight and dimensions and the maximum R-square for all characteristics were very small. Fiber diameter could be predicted using the formula of multiple regression includes body weight and length and chest depth according to the maximum R-square ($P<0.01$). While Kemp fiber percentage could be predicted using the formula includes body weight, fleece weight and Shoulder height. Only the simple regression equation of Fleece weight on body weight was significant ($P<0.01$) with R-square (0.19). The correlation coefficient was done among all studied characteristics and ranged between -0.69 ($p<0.01$) (between fine fiber and coarse fibers) and 0.94 ($p<0.01$) (between fine fiber and S/P ratio). It was concluded that wool properties of Arabi sheep were not at standard level especially in both fine and Kemp properties and need to be improved.

1. INTRODUCTION

The Arabi sheep as the other of local Iraqi sheep have the fat tail and the smallest local sheep in size, and they are producing coarse wool suitable for the carpet industry, and are raised in the southern and central regions of Iraq Al-Azzawi (1977). In the flat area of northern region, the farmers and

breeders began raising this breed because of fitness with areas as well for their small requirements in comparison with other locals Al-Azzawi (1977). That the specifications of good wool used in the carpet industry cannot be obtaining from sheep, so the process of mixing several types of wool to equalize the price is required in manufacturing qualities such as wool weakness and Al-Oramary (2002) already mentioned to the types of carpet wool used in the carpet mixing process in the carpet industry. The wool production is one of economic importance in some countries of the world such as Australia and New Zealand, and its study is not less importance than meat and milk production. This part of breeders income depends on the quantity and quality of wool produced. Wool production varies from year to year, these differences are mainly due to differences in seasons, and many studies have shown a significant seasonal variability in wool growth for sheep-producing such McFarlane, 1952; Champion and Robards, 2000; and Francis et al., 2000& and they reported that other factors affect wool growth than seasons, such as nutrition, pregnancy, and lactation. Ryder (1968) explained that the number of wool fibers does not change by type of nutrition, but changes in the amount of nutrition lead to a change in the length and diameter of individual fibers due to reduced skin area through malnutrition and also indicated the possibility of increasing the productivity of wool in a head sheep by providing good food when compared to the selection of non-wool producing sheep. Dalton and Rae (1978) noted that undernourishment of pregnant ewes reduces the growth of wool in winter and inadequate nutrition during pregnant reduces the growth of wool in the spring. The regression procedure using maximum R-square improvement to carry out for selecting the best prediction equation for physical wool properties instead of measuring through instruments or manually, and linear regression using General Linear Model (GLM) procedure was applied to develop the prediction equation for physical wool properties.

Due to the lack of studies on wool production and its price in Arabi sheep as well lacks of breeding and improvement programs, and the absence of accurate data for physical properties of wool, this study was conducted to study the physical wool characteristics including staple and fiber length and weight of raw wool and the percentage of clean wool and the fiber type ratio and the ratio of (S/P) and fiber diameter and the effects of age of ewe and the sex of their lambs. Also obtaining the best equations to predict wool production and select sheep with more efficient in producing wool by turning food into Wool.

2. MATERIALS AND METHODS

2.1. Experimental Animals

This study was conducted in private field in Al-Murtkah village in Erbil during May 2017 and includes 72 ewes at different ages (2.5 to 5.5 years). Feeding ewes was based on pasture grazing in the spring and on the harvests of the wheat, with a limited amount of flour in winter in addition to hay. The weight of raw fleece was taken directly after the shearing and the samples were taken in May with the shearing processes in the Mid-Side as the best area for animal wool sampling (Taddo et al., 2000). The shearing processes were done manually.

Preparation of samples and laboratory measurements:

The staple length: (5) randomly staples were taken from each sample of the raw wool

samples to measure their lengths by using the normal ruler (Von Bergen, 1963).

Fiber length: The lengths of the fibers done manually by the normal ruler of 100 fibers were randomly measured from each sample of clean wool samples (single staple) based on (A.S., 1978).

The percentage of clean wool: It was calculated according to Chapman (1960), but by drying the entire woolen samples by drying oven rather than putting in the ventilation room (20 ± 2 °C and relative humidity 65 ± 2%), then followed washing by non-ionic detergent powder (SANDOSIN) and then dry the samples completely again instead of regaining the moisture as before washing process. The result was calculated according to the following equation. The samples were then used for physical measurements.

$$\text{The percentage of clean wool} = \frac{\text{Weight of the clean wool sample}}{\text{Weight of raw wool sample}}$$

Fiber diameter: A staple of each sample of clean wool was taken and placed in the ventilator room sufficiently for the purpose of regaining moisture to measure the fiber diameter (micron) of the rate (170 ± 10 fiber) per sample by Projection Microscope and according to the method described by (A.S., 1978).

Fiber type ratio: A staple was taken from each sample of clean wool to calculate the ratio of each fiber type, coarse and fine wool and kemp by weight (Chapman, 1960). The ratio of (S/P) was calculated as the ratio of fine fibers to (coarse and kemp) fibers.

Statistical Analysis: data were statistically analyzed using the statistical program (SAS, 2002-2003) to study the effect of ewes age and the sex of the lambs on the characteristics of weight of wool and raw and clean wool, staple and fiber length, fiber type ratio and fiber diameter depending to the following general linear model.

$$Y_{ijk} = \mu + A_i + B_j + e_{ijk}$$

Where: Y_{ijk} is the value of jth observation, μ over all mean, A_i the effect of ewe age (2.5 to 5.5 years), B_j the effect of lamb sex (male or female) and e_{ijk} is the experimental error.

Duncan Multiple Rang Test within SAS (2002-2003) used for comparison between means within each group factors.

Several multiple regression equations among the studied traits were carried out using Maximum R-square Improvement procedure which is usually the criterion to select the best prediction equations (Draper and Smith, 1966). Also simple linear regression equations and correlation coefficients were obtained between each pair of wool properties and body weight using the program SAS (2002-2003).

3. RESULTS AND DISCUSSION

The raw fleece weight:

The overall mean weight of the raw fleece was 1653.75 g (Table 1) and was closely correlated with the Al-Oramary (2002) who reported that the highest and lowest weight of the raw fleece weight for some study of Kurdi sheep were 2.10 and 1.49 kg and for Hamadani sheep 2.34 and 1.83 kg respectively. Aziz (1991) recorded 2.15 kg in Kurdi sheep, also Khoshnaw (2007) and Khoshnaw and Hussein (2013) found that the raw fleece weights were 2.19 and 2.303 kg in Hamadani and Kurdi sheep. The results showed that age of ewes affect the raw fleece weight significantly ($p \leq 0.05$) and the ewes aged 5.5 years were higher (1748.23 g) than ewes aged 3.5 years (1532.35 g). This result is consistent with Al-Oramary (2002) who showed that ewes at ages 1, 2 and

3 years significantly exceeded the ewes at age 4 and 5 years. Whereas the results were in contrast with those found by Aziz (1991) and Khoshnaw (2007). The effect of lamb sex was non-significant on the fleece raw weight, and the ewes born females had a lower fleece raw weight comparing with those born males reported (1639.35 and 1664.63 g, resp.). This result was similar with Al-Azzawi (1977) and Taddo et al. (2000) in terms of non-significant effect of type birth on raw fleece weight in Hamadani ewes.

Percentage of clean wool:

The overall mean of clean wool percentage was 79.06% (Table 1). This result was similar to that of Aziz (1991) in Kurdi sheep (76%) and Al-Oramary (2002) in Hamadani sheep (79.29%), and higher than Ashmawy and Al-Azzawi (1980) in the same breed (53.88%) and Al-Azzawi (1977) in Awassi sheep (54.53%) and Khoshnaw and Hussein (2013) 56.67%. As shown in table (1) no significant effect appeared in the wool clean percentages according to age of ewes; and this is consistent with what found by Khoshnaw (2007) Al-Oramary (2002) in Hamadani sheep. Also there were no significant differences between the ewes born males or females and the percentages of their clean wool were 80.07 and 77.72% respectively.

Fiber diameter:

Table 1 shows that the overall mean of the fiber diameter is 28.24 microns, which is close to those obtained earlier by Al-Azzawi (1977) and Al-Oramary (2002) and Khoshnaw (2007) in Hamadani sheep and by Khoshnaw and Hussein (2014) in Karadi sheep and were 29.97, 28.40, 31.27, and 30.73 microns respectively, and less than those found by Ma'arof *et al*. (1989), Aziz (1991) and Al-Oramary (2002) (37.19, 37.09 and 38.4 microns resp.). Although ewes with 4.5 years old had the highest fiber diameter, age of ewe had no significant differences (Table 1), and this result was in consistent with that of Al-Oramary (2002). Also the sex of lamb did not affect the fiber diameter significantly; anyway it was coarser (29.39 microns) in ewes born female lambs compared to those born male lambs (27.36 microns). These result was similar to that of Khoshnaw (2007) in Hamadani ewes who claimed to non-significant effect of sex on fiber diameter and ewes born female had coarser fiber compared with ewes born male (29.95 and 27.83 microns, resp.).

Crimps:

Table (1) shows that the overall mean of number of (crimps/cm) of the length of the fiber were 1.33 crimps / cm. This result is in agreement with that of Khoshnaw (2007) 1:31 crimps / cm in Hamadani ewes, while it was lower compared with Ma'arof et al (1989) in Hamadani sheep (2.29 crimps / cm) and higher compared with Al-Oramary (2002) in Hamadani sheep (0.63 crimps / cm). Ewe’s age group had no significant effect on crimps/cm. While Khoshnaw (2007) found that ewes aged two years had significantly higher number of crimps/cm in Hamadani ewes comparing with ewes aged 3 years and in-addition it was noticed that the number of crimps decreased by age up to 5 years and then increased. The impact of sex of lamb was not significant on the number of crimps/cm which was 1.29 and 1.36 crimps / cm in ewes born females and male lambs respectively. The lack of significant of lamb sex on the number of crimps / cm agreed with Al-Oramary (2002).

Staple length:

Table (٢) shows that the overall mean of staple length is 9.36 cm which is similar to that of Khoshnaw and Hussein (2014) (9.87 cm) in Kurdi sheep, while less compared to the results of each of Ma'arof et al. (1989), Al-Oramary (2002), Aziz (1993) and Khoshnaw (2007) (11.89, 12.03, 12.51 and 11.27 cm, resp.) in Hamadani sheep and Aziz (1991) in Kurdi sheep (11.8 cm). The effect of ewes age on staple length was not significant (Table 2) and ewes aged 5.5 years had shorter staple (8.82 cm) compared with ewes at other ages, which were close to each other. The non-significant differences in staple length among age groups of Kurdi and Hamadani sheep were found earlier by Aziz (1991), Al-Oramary (2002) and Khoshnaw (2007) respectively. It was found that sex of lamb didn’t affect staple length significantly, where the ewes

born males or females were 9.52 and 9.15 cm resp. This is in agreement with Khoshnaw (2007) in Hamadani ewes during the two periods of the year.

Fiber Length:

From table (2), it appears that the overall mean of fiber length (16.03 cm) and this is higher than those found by Khoshnaw (2007) (14.65 cm) and Khoshnaw and Hussein (2014) (14.76 cm) in Kurdi sheep, while it was lower than each of Al-Azzawi (1977) and Al-Oramary (2002) in Hamadani sheep (18.56 and 18.25 cm, resp.). The same table shows that the effect of ewe's age is non-significant in fiber length and this is similar in comparison to Khoshnaw and Hussein (2014) in Kurdi sheep. Al-Oramary (2002) also obtained the same result with the superiority of ewes at 1 year of age on ewes with other ages and pointed to significant differences among different ages. An earlier showed a significant effect of the age of ewes on the fiber length (Al-Azzawi, 1977). The effect of the lamb sex was not significant in fiber length and ewes born female were larger in fibers length (16.18 cm) while ewes born male had a lower length of fiber (15.91 cm). This result is counterproductive with the results of Khoshnaw (2007) who found a significant effect of lamb sex ($p \leq 0.05$) on the fiber length.

Fiber types ratio:

1. **Fine fiber ratio:**

Table (3) shows that the overall mean of fine fiber is 52.42%. The result obtained in this study was higher than those found by Ma'arof *et al.* (1989), Al-Oramary (2002) and Khoshnaw (2007) (36.74, 28.42 and 41.41%, respectively.) in Hamadani sheep, as well by Khoshnaw and Hussein (2014) in Karadi sheep (43.88%), while the result was less than that recorded by Al-Azzawi (1977) in Hamadani sheep (56.34%). From the table (3), it was noticed that ewes age affected fine fiber ratio significantly ($p \leq 0.01$) where ewes aged 2.5 and 3.5 years had the largest percentages (55.10 and 61.61%, respectively), whereas Khoshnaw and Hussein (2014) found that the effect of ewes age on fine fiber was non-significant. Also, many earlier researchers found that there was no significant effect of the age of ewes in the percentage of fine fibers ratio (Ma'arof *et al.*, 1989; Al-Oramary, 2002; and Khoshnaw, 2007) in Hamadani sheep. It was found that the effect of the lamb sex is not significant on the fine fiber ratio and is consistent with Khoshnaw (2007) in Hamadani sheep.

2. **Coarse fiber ratio:**

The overall mean of coarse fiber obtained in this study was 38.89% (Table 3) and was lower than those recorded earlier in Hamadani sheep by Al-Azzawi (1977); Ma'arof et al. (1989); Al-Oramary (2002); Khoshnaw (2007); and in Karadi sheep by Khoshnaw and Hussein (2014) which were 49.49, 64.21, 59.37, 41.41 and 49.97 % respectively. From the same table it is noted that the differences in coarse fiber percentages of ewes having different ages were significant ($p \leq 0.05$) and the ewes aged 5.5 years surpassed those aged 3.5 years (43.73 vs. 31.65%). This result was in contrast with those found by Khoshnaw (2007), Al-Oramary (2002), and Khoshnaw and Hussein (2014) who found non-significant differences in coarse fiber according to different ages of ewes. Although ewes born male lambs had highest coarse fiber (40.08%) than that born female lambs (37.32%) but the differences were not significant.

3. **Kemp fiber ratio:**

This study revealed that the overall mean of kemp fiber was 8.68% (Table 3), which is close to the results of Ma'arof et al. (1989) and Khoshnaw (2007) (6.13 and 8.97% resp.) and higher than those of Al-Azzawi (1977) , Aziz (1993) and Al-Oramary (2002) in Hamadani sheep (2.3%, 2.39 and 2.3%, respectively) and Aziz (1991) in Kurdi sheep (2.3%).

It was found that ewes aged 4.5 and 5.5 years exceed those aged 2.5 and 3.5 years significantly ($p \leq 0.01$) in their kemp fiber percentages and were 12.04, 8.19, 6.35, and 6.73 % respectively. Also Khoshnaw and Hussein (2014) found the significant ($p \leq 0.05$) effect, and showed that ewes at the age of two years were highest (11.16%) and the lowest for ewes at one year (4.06%). While Aziz (1991) and Al-Oramary (2002) did not found any significant differences in Hamadani sheep. The effect of lamb sex was significant ($P \leq 0.05$) and the ewes born female had higher percentage (11.12%) than ewes born males (6.83%).

4. **(S/P) ratio:**

Table (3) shows that the overall mean of (S/P) ratio is (1.27), which is higher than (0.915) found by Khoshnaw and Hussein (2014). The effect of age of ewes on (S/P) ratio was significant (p≤0.01) and ewes aged 2.5 and 3.5 years had the highest ratio, while Khoshnaw and Hussein (2014) not found the significant effect of age of ewes, and they noted decreasing the ratio with advanced ages in which be extrusive with fine fiber ratio and inverse with coarse fiber ratio, and the mean the S/P ratio will increase by increasing of fine fibers. The lower (S/P) ratio is better in the carpet industry, unlike the textile industry. The effect of the lamb sex born was non-significant in this property.

Multiple Regressions:

Through tables 4 to 13 shows the ordering with each set of physical wool properties combination according to the R-square values. In view of results, for raw fleece weight entering new additional trait did not improve the predicted raw fleece weight although R-square increased from 0.195 to 0.270 in step 5, so it could be predicted depending on body weight only (equation-1). Also to predict clean wool percentage, the R-square increased in 6 steps from 0.08 to 0.096 only which mean it could be predicted depending on Heart girth (equation-1). While in predicting fiber diameter from 0.083 to 0.252 in step 3 (equation-3), and the best equation to predict fiber diameter is depending on body weight, body length and chest depth (Table 6).

To predict staple length, fiber length, crimp (numbers/cm), fine fiber percentage, coarse fiber percentage, and S/P ratio, the maximum R-square increased slightly with additional parameters that it couldn't depending on those predicted traits. The prediction equations of kemp fiber percentage R-square improved from 0.072 to 0.231 in the third step, which mean that the best prediction equation for kemp fiber % will depend on body weight, fleece weight and Shoulder height. From the above results obtained in this study, the R-square is too low for most of the studied traits, so it will be better to investigate if there were other parameters affect the studied traits significantly and could be used in obtaining the best regression equation with more precession and reliable.

Simple regression

Table 14 shows the simple regression of physical wool properties on body weight, and the most of regressions were non-significant except that of raw fleece weight (p≤0.01), Kemp fiber percentage and fiber diameter (p≤0.05), and the values of R-square were slightly excluding that of raw fleece weight (0.19). however, the regression equations for physical wool properties on raw fleece weight were obtained (Table 15) and their values of R-square value of these trait had slight variation., The regression of staple length on raw fleece weight was significant (*P*≤0.05).

Correlation coefficient

Correlation coefficients between each pair of the studied traits were calculated and listed in Table 16. The most important positive correlation coefficients (p≤0.01) were those between clean wool% with fiber diameter (0.31), between fiber diameter and kemp fibers % (0.31), between crimp number with each of S/P ratio (0.48) and fine fiber% (0.49), between staple length with each of coarse fiber % (0.36) and fiber length (0.47) and between fine fiber % with S/P ratio (0.94), the positive correlation mean increasing and improving one trait will increase and improve the other trait. Whereas the negative correlation means increasing one trait will decrease the other and in this study the most important negative correlations (p≤0.01) were found between fiber diameter with fine fiber % (-0.30), between crimp number/cm with each of coarse fiber % (-0.52) and fiber length (-0.33), between staple length with each of S/P ratio (-0.31) and fine fiber % (-0.35), between fiber length with each of kemp fiber % (-0.45) and coarse fiber % (-0.53), between fine fiber % with coarse fiber % (-0.69) and finally between coarse fiber % with each of S/P ratio (-0.64) and kemp fiber % (-0.62). The correlation coefficients among physical wool properties were obtained and discussed previously by several researchers including Al-Oramary (2002), Khoshnaw (2007), and Khoshnaw and Hussein (2013 and 2014) in Kurdi and Hamadani sheep.

Conclusion:

From present study can be conclude that Arabi sheep are one of the native breeds producing wool carpets, but the qualities were not at the standard levels of carpet industry because of the lack of interest by the farmers and breeders in producing wool because of its low price.

The values of R-square for prediction equations were slight variation for each of the physical wool properties on body and raw fleece weight so it will be hardly to be used.

Age of ewes and sex of lamb were non-significant on most wool characteristics. Although the maximum R-square slightly increased gradually in the equation to predict most of traits by depending body weight only.

Recommendation:

It could be recommended that farmers and breeders should be interest in improving the Arabi breed depending on their wool production.

Table (1) Means ± S.E. for the effect of ewes age and lamb sex on fleece raw weight, clean wool, fiber diameter and crimps

Factors Affecting	Number	Raw fleece weight (gm)	Clean wool %	Fiber diameter (micron)	Crimps/cm
Over all mean	72	1653.75 ± 29.67	79.06 ± 1.44	28.24 ± 0.66	1.33 ± 0.04
Ewe age		*	NS	NS	NS
2.5	15	1633.33 ± 53.37 ab	81.47 ± 2.86	27.18 ± 1.13	1.42 ± 0.06
3.5	17	1532.35 ± 62.41 b	77.69 ± 2.47	27.74 ± 1.00	1.46 ± 0.10
4.5	23	1686.96 ± 38.52 ab	82.28 ± 3.02	29.50 ± 1.49	1.22 ± 0.06
5.5	17	1748.23 ± 78.11 a	73.94 ± 2.61	27.94 ± 1.37	1.27 ± 0.10
Sex of lamb		NS	NS	NS	NS
Male	41	1664.63 ± 31.19	80.07 ± 1.99	27.36 ± 0.86	1.36 ± 0.06
Female	31	1639.35 ± 55.79	77.72 ± 2.08	29.39 ± 1.10	1.29 ± 0.06

* = ($p \leq 0.05$)

The different letters from the same column for the same factor is sign ($p \leq 0.05$)

Table (2) Means ± S.E. for the effect of ewes age and lamb sex on Staple and Fiber length

Factors Affecting	Number	Staple length (cm)	Fiber length (cm)
Over all mean	72	9.36 ± 0.27	16.03 ± 0.36
Ewe age		NS	NS
2.5	15	9.73 ± 0.48	16.94 ± 0.47
3.5	17	9.48 ± 0.86	15.04 ± 0.48
4.5	23	9.42 ± 0.98	15.64 ± 0.57
5.5	17	8.82 ± 0.27	16.94 ± 0.73
Sex of lamb		NS	NS
Male	41	9.52 ± 0.41	15.91 ± 0.45
Female	31	9.15 ± 0.35	16.18 ± 0.60

Table (3) Means ± S.E. for the effect of ewes age and lamb sex on Fiber type ratio (fine, coarse and kemp) and (S/P) ratio

Factors Affecting	Number	Fine fiber %	Coarse fiber %	Kemp fiber %	(S/P)
Over all mean		52.42 ± 1.36	38.89 ± 1.72	8.68 ± 1.23	1.27 ± 0.09
Ewe age		**	*	*	**
2.5	15	55.10 ± 3.09 a	38.55 ± 3.46 ab	6.35 ± 1.85 c	1.45 ± 0.24 ab
3.5	17	61.61 ± 2.96 a	31.65 ± 4.00 b	6.73 ± 1.69 c	1.87 ± 0.22 a
4.5	23	47.08 ± 1.63 b	40.88 ± 2.92 ab	12.04 ± 2.94 a	0.93 ± 0.07 c
5.5	17	48.08 ± 2.10 b	43.73 ± 3.14 a	8.19 ± 2.36 b	0.98 ± 0.10 bc
Sex of lamb		NS	NS	*	NS
Male	41	53.08 ± 1.88	40.08 ± 2.15	6.83 ± 0.96 b	1.33 ± 0.13
Female	31	51.55 ± 1.96	37.32 ±2.85	11.12 ± 2.53 a	1.20 ± 0.12

* = (p≤0.05), **= (p≤0.01)

The different letters from the same column for the same factor is sign. (p≤0.05)

Table (4) multiple regression equation using maximum R-square improvement fleece weight on (body weight and dimensions)

Steps	R-square	Intercept	Body weight	Body length	Heart girth	Chest depth	Shoulder height
1	0.195	353.41	31.84 **				
2	0.228	1085.43	29.30 **	- 10.15			
3	0.249	1326.70	35.53 **	- 8.52			- 7.29
4	0.270	1261.22	25.34 **	- 8.35	5.61		- 8.74
5	0.272	1152.28	24.24 *	- 7.74	5.68	3.33	- 8.87

* = (p≤0.05), **= (p≤0.01)

Table (5) multiple regression equation using maximum R-square improvement clean wool percentage on (fleece weight, body weight and dimensions)

Steps	R-square	Intercept	Body weight	Fleece weight	Body length	Heart girth	Chest depth	Shoulder height
1	0.080	46.25				0.41 *		
2	0.085	53.74				0.43 *	- 0.25	
3	0.089	61.31				0.46 *	- 0.24	- 0.15
4	0.092	58.42	0.26			0.40	- 0.31	- 0.17
5	0.095	48.36	0.29		0.14	0.40	- 0.26	- 0.19
6	0.096	51.02	0.34	- 0.002	0.14	0.42	- 0.25	- 0.21

* = (p≤0.05)

Table (6) multiple regression equation using maximum R-square improvement fiber diameter 0n (fleece weight, body weight and dimensions)

Steps	R-square	Intercept	Body weight	Fleece weight	Body length	Heart girth	Chest depth	Shoulder height
1	0.083	9.24	0.46 *					
2	0.173	20.18	0.62 **				- 0.52 **	
3	0.252	48.85	0.60 **		- 0.36 **		- 0.64 **	
4	0.282	42.75	0.52 **		- 0.41 **		- 0.65 **	0.20
5	0.291	39.65	0.44 *	0.002	- 0.39**		- 0.66**	0.21
6	0.296	39.15	0.37	0.002	- 0.38 **	0.06	- 0.65 **	0.20

* = (p≤0.05), **= (p≤0.01)

Table (7) multiple regression equation using maximum R-square improvement staple length on (fleece weight, body weight and dimensions)

Steps	R-square	Intercept	Body weight	Fleece weight	Body length	Heart girth	Chest depth	Shoulder height
1	0.052	13.11		- 0.002 *				
2	0.102	9.34		- 0.003 *		0.06		
3	0.122	5.64		- 0.002 *		0.04		0.06
4	0.124	6.33		- 0.002 *		0.04	- 0.02	0.07
5	0.126	8.32		- 0.002 *	- 0.02	0.04	- 0.03	0.07

6	0.126	8.26	0.005	- 0.002 *	- 0.02	0.04	- 0.03	0.07

* = (p≤0.05)

Table (8) multiple regression equation using maximum R-square improvement fiber length on (fleece weight, body weight and dimensions)

Steps	R-square	Intercept	Body weight	Fleece weight	Body length	Heart girth	Chest depth	Shoulder height
1	0.095	4.03						0.18 **
2	0.107	7.02	- 0.10					0.20 **
3	0.114	6.65	- 0.15			0.03		0.19 **
4	0.116	4.42	- 0.13		0.03	0.03		0.18 *
5	0.118	5.11	- 0.12	- 0.0005	0.03	0.04		0.18 *
6	0.118	5.38	- 0.12	- 0.0005	0.02	0.04	- 0.008	0.18 *

* = (p≤0.05), **= (p≤0.01)

Table (9) multiple regression equation using maximum R-square improvement (crimps/cm) on (fleece weight, body weight and dimensions)

Steps	R-square	Intercept	Body weight	Fleece weight	Body length	Heart girth	Chest depth	Shoulder height
1	0.051	0.26	0.01					
2	00.61	- 0.06			0.01		0.01	
3	0.063	- 0.30			0.003		0.01	0.01
4	0.063	- 0.23	- 0.002		0.003		0.01	0.01
5	0.064	- 0.28	- 0.004	0.00003	0.003		0.01	0.01
6	0.064	- 0.27	- 0.003	0.00004	0.003	- 0.00008	0.01	0.01

Table (10) multiple regression equation using maximum R-square improvement Fine fiber percentage on (fleece weight, body weight and dimensions)

Steps	R-square	Intercept	Body weight	Fleece weight	Body length	Heart girth	Chest depth	Shoulder height
1	0.055	78.13				- 0.32*		

2	0.066	67.83				- 0.32 *	0.34	
3	0.072	52.08			0.20	- 0.34 *	0.42	
4	0.075	56.79	- 0.26		0.19	- 0.28	0.48	
5	0.077	53.48	- 0.28		0.16	- 0.30	0.47	0.10
6	0.078	54.57	- 0.27	- 0.0006	0.16	- 0.29	0.48	0.10

Table (11) multiple regression equation using maximum R-square improvement Coarse fiber percentage on (fleece weight, body weight and dimensions)

Steps	R-square	Intercept	Body weight	Fleece weight	Body length	Heart girth	Chest depth	Shoulder height
1	0.052	-3.26						0.64
2	0.068	13.58					-0.53	0.68 *
3	0.102	1.68		0.009			- 0.69	0.71 *
4	0.125	- 3.64	- 1.26 *	0.01		0.39		0.70 *
5	0.137	6.15	- 1.09	0.01		0.38	- 0.48	0.71 *
6	0.137	9.47	- 1.09	0.01	- 0.04	0.38	- 0.49	0.72 *

* = (p≤0.05)

Table (12) multiple regression equation using maximum R-square improvement kemp fiber percentage on (fleece weight, body weight and dimensions)

Steps	R-square	Intercept	Body weight	Fleece weight	Body length	Heart girth	Chest depth	Shoulder height
1	0.072	- 24.30	0.80 *					
2	0.193	11.68	1.09 **					- 0.73 **
3	0.231	18.92	1.41 **	- 0.009				- 0.81 **
4	0.236	19.55	1.54 **	- 0.008		- 0.11		- 0.77 **
5	0.238	16.23	1.48 **	- 0.008		- 0.10	0.16	- 0.78 **
6	0.239	13.45	1.48 **	- 0.008	0.03	- 0.10	0.17	- 0.78 **

* = (p≤0.05), **= (p≤0.01)

Table (13) multiple regression equation using maximum R-square improvement (S/P) on (fleece weight, body weight and dimensions)

Steps	R-square	Intercept	Body weight	Fleece weight	Body length	Heart girth	Chest depth	Shoulder height
1	0.041	2.78				- 0.10		
2	0.068	1.66				- 0.02 *	0.03	
3	0.078	0.90				- 0.02 *	0.03	0.01
4	0.083	1.11	- 0.01			- 0.02	0.04	0.01
5	0.086	0.33	- 0.01		0.01	- 0.02	0.04	0.01
6	0.088	0.14	- 0.02	0.0001	0.01	- 0.02	0.04	0.01

* = ($p \leq 0.05$)

Table (14) Linear regression equation between physical wool properties and body weight

X	Y	Linear regression equation	R^2
Body weight	Fleece weight	Y= 353.41+ 31.84 X **	0.19
	S/P	Y= 2.36 + (- 0.02) X	0.03
	Kemp fibers %	Y= (- 24.30) + 0.80X *	0.07
	Coarse fibers %	Y= 43.55 + (- 0.11)X	0.007
	Fine fiber %	Y= 74.38 + (- 0.53)X	0.02
	Fiber length	Y= 16.99 + (- 0.02)X	0.0007
	Staple length	Y= 9.17 + 0.004 X	0
	Crimps	Y= 1.04+ 0.007 X	0.004
	Fiber diameter	Y= 9.24 + 0.46 X *	0.08
	Clean %	Y= 52.44 + 0.65 X	0.03

* = ($p \leq 0.05$), **= ($p \leq 0.01$)

Table (15) Linear regression equation between physical wool properties and fleece weight

X	Y	Linear regression equation	R^2
Fleece weight	S/P	Y= 1.52+ (- 0.0001) X	0.002
	Kemp fibers %	Y= 8.03 + 0.0003 X	0.0001
	Coarse fibers %	Y= 28.46 + 0.006 X	0.01
	Fine fiber %	Y= 61.00 + (- 0.005) X	0.01
	Fiber length	Y= 17.99 + (- 0.001) X	0.009
	Staple length	Y= 13.11 + (- 0.002) X *	0.05
	Crimps	Y= 1.29 + 0.00002 X	0.0002
	Fiber diameter	Y= 20.34+ 0.004 X	0.04
	Clean %	Y= 73.10 + 0.003 X	0.005

* = ($p \leq 0.05$)

Table (16) The correlation coefficient between physical wool properties

Steps	Fleece weight	Clean %	Fiber diameter	Crimps	Staple length	Fiber length	Fine fiber %	Coarse fibers %	Kemp fibers %
S/P	- 0.04	0.13	- 0.25 *	0.48 **	- 0.31 **	- 0.22 *	0.94 **	- 0.64 **	- 0.08
Kemp fibers %	0.009	- 0.003	0.31 **	0.21	- 0.08	-0.45 **	- 0.11	- 0.62 **	
Coarse fibers %	0.10	- 0.08	0.02	- 0.52 **	0.36 **	- 0.53 **	- 0.69 **		
Fine fiber %	- 0.11	0.13	- 0.30 **	0.49 **	- 0.35 **	- 0.21			
Fiber length	- 0.09	0.18	0.07	- 0.33 **	0.47 **				
Staple length	- 0.24 *	0.25 *	0.26 *	- 0.18					
Crimps	0.14	0.15	- 0.02						
Fiber diameter	0.21	0.31 **							
Clean %	0.07								

* = (p≤0.05), **= (p≤0.01)

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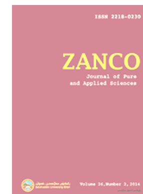

Effect of Adding Acetic Acid on Performance and Environmental Properties of Engine Diesel Fuel.

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A B S T R A C T

This paper addressed processing of the local Petroleum diesel fuel (PD) using glacial acetic acid (GAA). The process resulted good changes in the performance and environmental properties of the diesel fuel against its properties before the processing. The performance properties like flash point, cetane index, cetane number, and pour point, also the environmental properties like aniline point (indication of aromatics content) and total sulfur ratio were changed positively. Two layers were separated, in 10vol% GAA up to 5vol%GAA and the bottom layer decreased with decreasing of added glacial acetic acid. For 2vol%GAA, no bottom separated layer was observed. 2vol% GAA and high centrifugation speed (2000 rpm.) with room temperature and pressure operation condition ,also with decreasing the pour point , total sulfur percent , aromatics content ,and flash point , with somewhat a short production cycle (about 24 hours) is enough to increase the cetane number more than 15 degrees , with keeping the acidity without change , All of that decided the 2vol% GAA is the optimum point , although in this point the cloud point is effected negatively but it is under control and can be solved easily by chemical additives.

1. INTRODUCTION

The diesel engine is now fully established in a variety of applications on land and in marine use. On land, it serves to power trains, buses, trucks, and automobiles, and to run construction, petroleum drilling, agricultural, and other off-road equipment. At sea, it serves both to provide main propulsion power and to run auxiliaries (RAND , 2003). The major parameters which control the diesel quality and ranges of usage are the cetane number, aromatic content, density, sulfur content, distillation profile, viscosity, cloud and pour points, and flash point (JONES , PUJADÓ , 2006). Some of these parameters or properties are interconnected, for example, the saturation

of aromatics leads to an increase in cetane number. This fact led to UNISAR process for saturation of aromatics for naphtha, kerosene, and it applied for diesel fuels in 1990s when, the need to increase the cetane number of diesel fuel has grown. Increasing the cetane number also improves engine performance and decreases emissions. This process is with high yield but also with high cost (MEYERS ,2013).

Sometimes the quality and environmental problems are solved by mixing the petroleum diesels with chemical Additives. To improve the diesel fuel performance, Cetane number improvers such as alkyl nitrates and nitrites can improve ignition quality. Pour-point depressants can improve low-temperature performance. Antismoke additives may reduce exhaust smoke (related proportionally to aromatics content), which is a growing concern as more and more attention is paid to atmospheric pollution. Antioxidant and sludge dispersants can minimize or prevent the formation of insoluble compounds (RAND, 2003).

Using of glacial Acetic acid to process the used lubricating oil is adopted by some researchers and they got comparable results in comparison with traditional processing units (HAMAWAND *et al.*, 2013). This encouraged us to try using glacial acetic acid to improve local diesel fuel properties depending on its neighborhood and similarity to the lubricating oil cut (base oil) in main distillation unit (SPEIGHT ,2015).

Acetic acid is regarded as a weak carboxylic acid with a pungent odor that exists as a liquid at room temperature. Pure acetic acid is often called glacial acetic acid because it freezes slightly below room temperature at 16.7°C. When bottles of pure acetic acid froze in cold laboratories, snow like crystals formed on the bottles; thus, the term glacial became associated with pure acetic acid (MYERS ,2007).

2. MATERIALS AND METHODS

2.1 Diesel fuel properties test

The parameters or properties were depended to compare the diesel fuels are as following :

- Flash Point

The flash point test determines the temperature at which the vapors from petroleum oil will ignite or "flash". To determine the flash point, a sample is heated at a specified rate and an open flame introduced into the emitted vapors at specified time intervals. The. temperature at which the vapors ignite is recorded as the flash point. The open cup method is often used because of its simplicity. Below flash points of 510 ^{o}F (266 ^{o}C), the open cup flash point is approximately 10 ^{o}F (5.6 ^{o}C) higher than the closed cup flash point for the same sample. The open cup method heats the oil at a rate of 10 ^{o}F (5.6 ^{o}C) per minute with the test flame applied at 30 second intervals. Therefore, the best accuracy that could be expected from this method is plus or minus 5 ^{o}F (2.8 ^{o}C). The closed cup tester heats the oil at a rate of 1.8 ^{o}F per minute and is the more precise of the two methods (KAES ,2000). ASTM D 6450, Standard Test Method for Flash Point by Continuously Closed Cup (CCCFP) Tester, can also be used but ASTM D 93 was the referee method (RAND ,2003).

- Pour Point

The pour point test (ASTM D97) is useful in determining the waxiness of petroleum oil. A sample of oil is first heated to 115 ^{o}F (46.4 ^{o}C) to ensure that all wax is in solution. It is then cooled to 90 ^{o}F (32.6 ^{o}C). The sample is now placed in a cooling bath that is maintained from IS to 30 ^{o}F (8.3 - 16.7 ^{o}C) below the pour point of the oil. The test jar containing the oil is removed from the bath at 5 ^{o}F (2.8 ^{o}C) intervals and tilted to determine if the oil will flow. If no

movement is detected for 5 seconds the solid point has been reached. The pour point is defined as the temperature 5 ^oF (2.8 ^oC) above the solid point. The same procedure is used to determine the cloud point of petroleum oil. The cloud point is defined as the temperature at which a distinct cloudiness or formation of crystals appears in the bottom of the test jar. The cloud point is typically about 5 ^oF (2.8 ^oC) higher than the pour point (KAES ,2000)

- Cetane Number and Cetane Index

The cetane number is used to measure the performance of a fuel in a diesel engine (KAES ,2000). The ignition properties of diesel fuels are expressed in terms of cetane number or cetane index. These are very similar to the octane number (except the opposite) and the cetane number expresses the volume percent of cetane ($C_{16}H_{34}$, high-ignition quality) in a mixture with alpha-methyl-naphthalene ($C_{11}H_{10}$, low ignition quality). The fuel is used to operate a standard diesel test engine according to ASTM test method D-613. Since many refineries do not have cetane test engines, a mathematical expression developed to estimate the cetane number is used. The number derived is called the cetane index and is calculated from the mid–boiling point and gravity of the sample, and it is actually an expression of the hydrogen/ carbon ratio of the hydrocarbon components in the sample; the higher the H/C ratio, the better the burning characteristics (i.e., the higher the smoke point and the higher the cetane index). It is also an indirect indicator of the aromatic content of the diesel fuel. Therefore, frequently a minimum cetane index specification is used as an alternative to maximum aromatics content. Lowering sulfur and aromatics contents specifications also lowers the particulate emissions from diesel engines (GARY *et al.*, 2007).

- Density, Specific Gravity

Density is the mass per unit volume of a substance. Density is temperature dependent. It is an important property of petroleum and petroleum products. The density related properties of petroleum and petroleum products are often used: (i) specific gravity and (ii) American Petroleum Institute (API) gravity. Specific gravity (relative density) is the ratio, at a specified temperature, of the oil density to the density of pure water. The API gravity scale (presented as °API) arbitrarily assigns API gravity of 10° to pure water. Thus (SPEIGHT ,2015):

$$API = \left(\left(141.5/\text{Specific gravity at } 15.6\,^\circ C \;-131.5\right)\right)$$

- Sulfur Content

The total sulfur content of diesel fuel can be determined in accordance with ASTM D4294 (standard test method for sulfur in petroleum products by energy dispersive X ray fluorescence spectroscopy). This method is applicable to both volatile and nonvolatile petroleum products with sulfur concentrations ranging from 0.015 to 5 mass percent (SPEIGHT ,2015 and, NADKARNI ,2007)

- Aniline Point and Mixed Aniline Point

This test method for the determination of aniline point and mixed aniline point is used with the fuel oils (ASTM D611, IP 2) helps in the characterization of pure hydrocarbons and their mixtures and is most often used to estimate the aromatic content of naphtha. Aromatic compounds exhibit the lowest aniline points, and paraffin compounds have the highest aniline points, with cycloparaffins (naphthenes) and olefins having aniline points between the two extremes (SPEIGHT ,2015). The aniline point is the lowest temperature at which the fuel is miscible with an equal volume of aniline and is inversely proportional to the aromatic content (RAND,2003).

- Calculated Properties

Attempts to estimate some properties of petroleum cuts by the imperial relations depending on other properties are acceptable and they may be take a form of practical or empirical equation or charts (RIAZI ,2005) for diesel fuels, the diesel index is more famous one which it estimated by specific gravity and aniline point of diesel fuel and it was important in diesel fuels rating. The first widely used measure of ignition quality was the diesel index. And it was calculated as (RAND,2003 and RIAZI ,2005):

Diesel Index = [(API Gravity)(Aniline Point in ^{o}F)] / 100

Also there are some empirical equations to estimate aromatics content which developed by Jenkins-Walsh, they developed this simple relation in terms of specific gravity and aniline point in the following form:

$$\%H = 11.17 - 12.89SG + 0.0389AP$$

Where, AP is the aniline point in Kelvin, by using %H estimated by previous equation to estimate aromatics percent by:

$$\%A = 233.54 - 15.67(\%H) \quad ,$$

where %A is Aromatics content (RIAZI ,2005).

2. 2 Experimental Apparatus and Procedure

For the purpose of this study a series of random experiments carried out on a local petroleum diesel fuel which it purchased by T-TOPCO Company from local market on December 2015, and its sample was sent to laboratories of Natural Recourses Ministry in Erbil city –Kurdistan region to test. In order to confirm the maximum ability of GAA to enhance the performance and environmental properties of this local diesel fuels, the first try was carried out by adding a (10 vol%, (10 ml of GAA and 90 ml of PD)) in a beaker. This sample mixed at 300 rpm for quarter hour up to 20°C on a hot plate magnetic stirrer. The beaker was open to the atmosphere as shown in figure (1).

The resulted mixture was centrifuged immediately for a quarter hour at different speeds of (600and 900 rpm.), then the mixture let to settle for different time intervals (15 minutes, 24 hours, and 96 hours) the color became less transparent and two separated layers were created. In fact, no color change was noted after 24 hours up to 96 hours but the real left time was recorded. In general, the bottom layer was increased with increasing the left time.

These set of procedures was repeated with rest volumetric ratio of acetic acid. For (5vol % GAA), the color is kept its transparency in comparison with (10% vol GAA with PD), but with smaller bottom layer, then it centrifuged at 900 rpm. , and let to settle for 24hours. Same set of procedures was repeated again and for (2% vol GAA and 98 %vol PD). The centrifugation speed was changed to 2000 rpm and left time stilled 24 hours. The treated mixture was kept its transparent level (color) as the same of preceding volumetric ratios of GAA, but without separated layers.

2.3 Experimental Apparatuses, Chemicals, Glassware

The apparatuses which used in this research divided to two groups:

2.3.1 Preparation apparatuses

1- Glacial acetic acid, purity 99.5%, Merck Inc., Germany

2- Local diesel fuel sample.

3- Graduated cylinder, 100 ml.

4- Graduated beaker, 100 ml.

5- Thermometer.

6- Hot plate magnetic bar stirrer –Stuart SB 162, UK.

7- Centrifuge separator, Fenam excelsa3 , mod280, Brazil.

2.3.2 Sample test apparatuses

<u>1-specific gravity, hydrometer with water bath, ASTM D1298</u>

Tested by: A2LA Traceable Hydrometers, Alla France Inc., France.

Test summery

The sample is brought to the prescribed temperature and transferred to a cylinder at approximately the same temperature. The appropriate hydrometer is lowered into the sample and allowed to settle. After temperature equilibrium has been reached, the hydrometer scale is read, and the temperature of the sample is noted. If necessary the cylinder and its contents may be placed in a constant temperature bath to avoid excessive temperature variation during the test [10].

2- Flash Point, ASTM D6450 (CCCFP).

Tested by: ERAFLASH, Eralytics Inc., Austria

Test summery

The lid of the test chamber is regulated to a temperature of at least 18° C below the expected flash point. A 1-mililiter test specimen is introduced into the sample cup, ensuring that both specimen and cup are at a temperature of at least 18° C below expected flash point, cooling if necessary. The cup is then raised and pressed onto the lid of specified dimensions to form the continuously closed but unsealed test chamber with an overall volume of 4 mL. The lid is heated at a prescribed, constant rate. An arc of defined energy is discharged inside the test chamber at regular intervals. After each ignition, 1.5 mL of air is introduced into the test chamber to provide the necessary oxygen for the next flash test. After each arc, the instantaneous pressure increase above the ambient barometric pressure inside the test chamber is monitored. When the pressure increase exceeds a defined threshold, the temperature at that point is recorded as the uncorrected flash point (NADKARNI ,2007).

3-Total Sulfur, ASTM D4294-03.

Tested by: Total Sulfur meter, RX-360SH, TANAKA Inc., Japan.

Test summary

The sample is placed in a beam emitted from an X-ray source. The resultant excited characteristic X radiation is measured, and the accumulated count is compared with counts from a previously prepared calibration standard to obtain the sulfur concentration. The measured concentrations range of this apparatus is 0 to 6.0wt%.

4- The Color, ASTM D6045.

Tested by: Tintometer PFX 680, LoviBond Inc., France.

5-Cetane number and cetane index.

Tested by: Portable near-infrared octane/cetane analyzer, ZX-101XL , Zeltex Inc., USA.

6- Pour and cloud points, ASTM D97 and D2500.

Tested by: Semi-automatic pour and cloud points apparatus, LP4/1, Lab Plant Inc., UK.

Test summary

The sample is first heated to a temperature above the expected cloud point and then cooled at a specified rate and examined periodically. The temperature at which haziness is first observed at the bottom of the test jar is recorded as the cloud point.

After preliminary heating, the sample is cooled at a specified rate and examined at intervals of 3°C for flow characteristics. The lowest temperature at which the movement of the diesel fuel is observed is recorded as the pour point. (NADKARNI ,2007).

7- Aniline point, ASTM D611-Method E.

Tested by: Automatic Aniline point apparatus, K10200, Kohler Inc., USA.

Test summary

Equal volumes of aniline and diesel fuel are mixed and heated until a miscible mixture is formed. On cooling at a prescribed rate, the temperature at which the mixture becomes transparent and clear is recorded and identified as the aniline point [1,3]

8-Acidity, ASTM D1093.

Tested by: PH meter and sunflower paper, Merck Inc., Germany.

Test summary

A sample is shaken with water and the aqueous layer is tested for acidity using methyl orange indicator (red color) (NADKARNI ,2007).

3. RESULTS AND DISCUSSION

The results of this research divided to two tables, the first is containing the direct tests measurements, and the second is containing calculated properties which depended on the values from table (1) then calculated by empirical relations from the references.

The specific gravity was showed stable behaviors and changed slightly (max. 1.5%). This slight increase was

10vol% of GAA with short left time (half hour) and slower centrifugation speed (600 rpm.) Impacted the total sulfur ratio and decreased it up to 19 weights %, while more left time (96 hours) lowered it to 14.5 mass %. Less GAA vol. % showed less impact on removed total sulfur mass ratios, even though, the centrifugation speed was increased.

Samples' colors were changed to the less transparent after adding acetic acid but it kept this transparent level during all tests.

The cloud points have affected negatively, and 10vol. % of GAA showed the worst cloud point result (18°C). This result might belong to the effect of low freezing point of GAA (16.7^{o}C). Other vol. % of GAA affected it slightly and it can be controlled by adding cloud point depressants (diesel fuel additives).

10 vol% GAA mixture showed negative impact on pour point value. Also, it can have related to low freezing point of GAA. Another vol. % of GAA impacted positively the pour point.

The adopted acidity test routine is similar to ASTM D1093. Figure (3) showed that the acidity effect was vanished in 2vol% GAA. This might have indicted happening a chemical reaction which consumed all GAA vol % and converted it to non –acidic products.

The flash point was most sensitive property to the added %vol GAA. The 2 vol. % GAA was enough to decrease its value by 11°C. The maximum decrease was with 10 vol. % which produced up to 20 ^{o}C. More left time was resulted more decrease in flash point.

The cetane number and cetane index were decreased with increase of vol. % GAA, reached 4 degrees in 10 vol. % GAA for cetane index and 3 degrees in cetane number. Lower vol. % GAA and higher centrifugation speed (2 vol. %, 2000rpm.) increased and reached up to 7.8 degrees for cetane index and 15.6 degrees for cetane number.

The aniline point (related inversely to aromatics content) was increased when vol. % GAA increased up to 8% (6.7% decreases in aromatics content) for 10 vol. % GAA. Only the Vol. % GAA was affecting this property.

4. CONCLUSIONS

The glacial Acetic acid effected on local diesel properties and enhanced its performance (like flash point, cetane index, cetane number, and pour point), and environmental properties (like aniline point (indication of aromatics content) and total sulfur ratio), although the processing was at room conditions.

In fact, all petroleum diesels must be between upper and lower limits of local quality laws to be usable. The big increasing of some properties like flash point and cetane number has two opposite impacts. The first is lower flash point, less ignition quality problem and this is the positive side. The second is less flash point, more storing problems and less safety, and this is a negative side. Judging flash point results must be undergo local fuel quality laws. best mixture was (2vol% GAA +PD) mixture which has more reasonable flashpoint and with acidity increase. Blending the last mixture with

heavier petroleum cuts or heavier petroleum diesels will give it an economical value.

Same problem was present with cetane number grades. Checking the last figure showed that (2vol% GAA +PD) raised the cetane number up to 69. The problem here, all diesel fuel engines designed to work on cetane number range (45-55). To overcome this problem, same solution that was suggested for flash point will suggested here, and it to blend the (GAA +PD) mixtures with heavier petroleum products or heavier bio or petroleum diesel. Some properties changed proportionally with GAA percent, but the (2 vol% GAA +PD) was the optimum. Each diesel physical property related to existence and percent of certain hydrocarbons or non-hydrocarbon components in the petroleum diesel. Reducing total sulfur content and aromatics content indicated happening a chemical reaction. Approving that will be our future research work.

Conflict of Interest:
There is no conflict of interest.

TABLES

Table (1): Direct measured properties of diesel fuel mixtures.

The Sample	Centrifugation speed (rpm)	left time (hours)	Specific gravity (at15.56°C) ASTM D1290	Flashpoint (°C) ASTM D6450	Total Sulfur wt% ASTM D4292	The Color ASTM D6045	Cetane Index	Cetane Number	Aniline point °C ASTM D611-Method E	cloud point °C ASTM D2500	pour point °C ASTM D97	Acidity ASTM D1093
Diesel fuel only			0.8284	61.3	0.6769	0.6	56.3	53.8	73.1	-1	-4	4.5
90% Diesel +10% Acetic Acid	600	0.5		43.3	0.5482							
90% Diesel +10% Acetic Acid	900	96	0.841	40.9	0.5787	0.8	52.2	50.9	78.9	18	-2	2.9
95%Diesel +5% Acetic Acid	900	24	0.832	44.9	0.6263	0.8	59.6	59.2	76.6	5	-6	4.25
98%Diesel +2% Acetic Acid	2000	24	0.8293	50.2	0.6492	0.8	64.1	69.4	74.9	4	-6	4.5

Table (2): The calculated properties of diesel fuel mixtures.

The Sample	Centrifugation speed (rpm)	left time (hours)	Specific gravity (at15.56 °C) ASTM D1298	Aniline point °C ASTM D611- Method E	API° gravity	Calculated %Aromatics content	Calculated Diesel Index
Diesel fuel only			0.8284	73.1	39.31	14.81	64.31
90% Diesel +10% Acetic Acid	900	96	0.841	78.9	39.13	13.89	65.27
95%Diesel +5% Acetic Acid	900	24	0.832	76.6	38.57	13.40	65.53
98%Diesel +2% Acetic Acid	2000	24	0.8293	74.9	36.75	13.82	63.96

FIGURE CAPTIONS

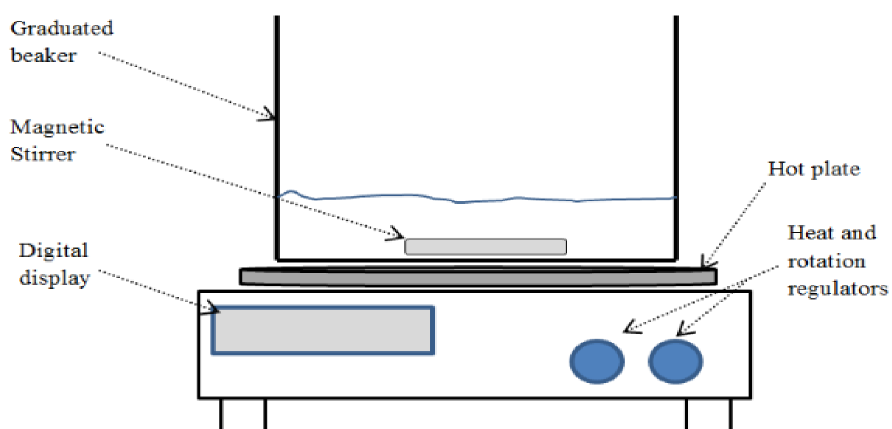


Figure (1): Schematic diagram of the test rig.

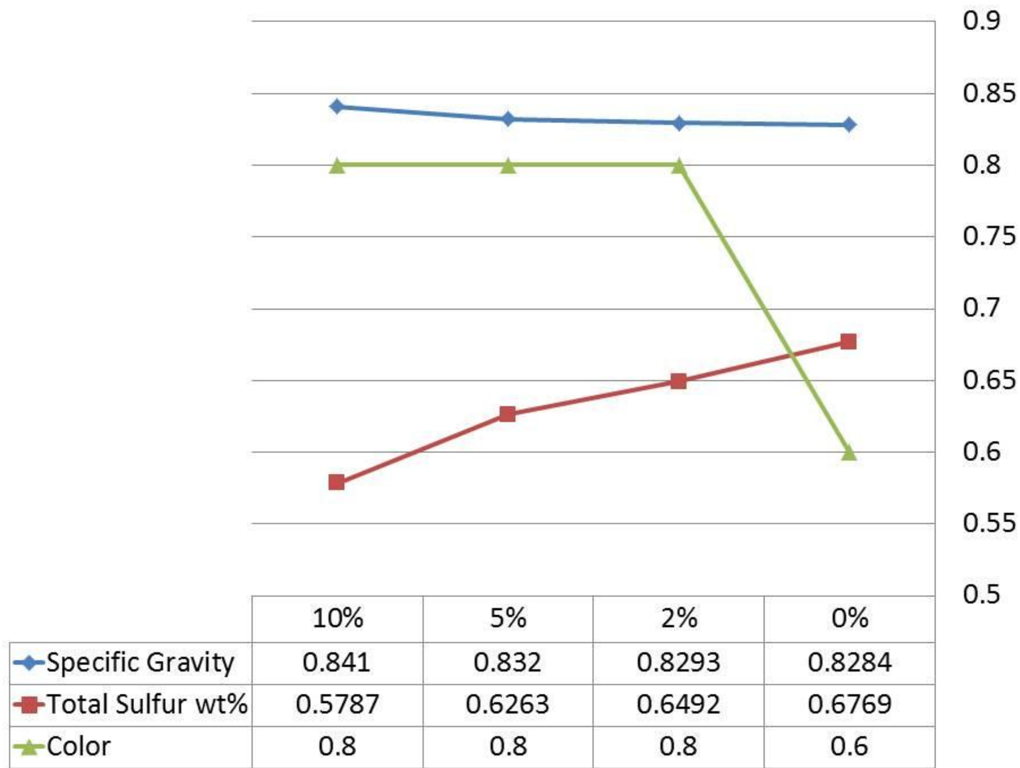


Figure (2): Diesel fuels properties vs. %vol. of GAA.

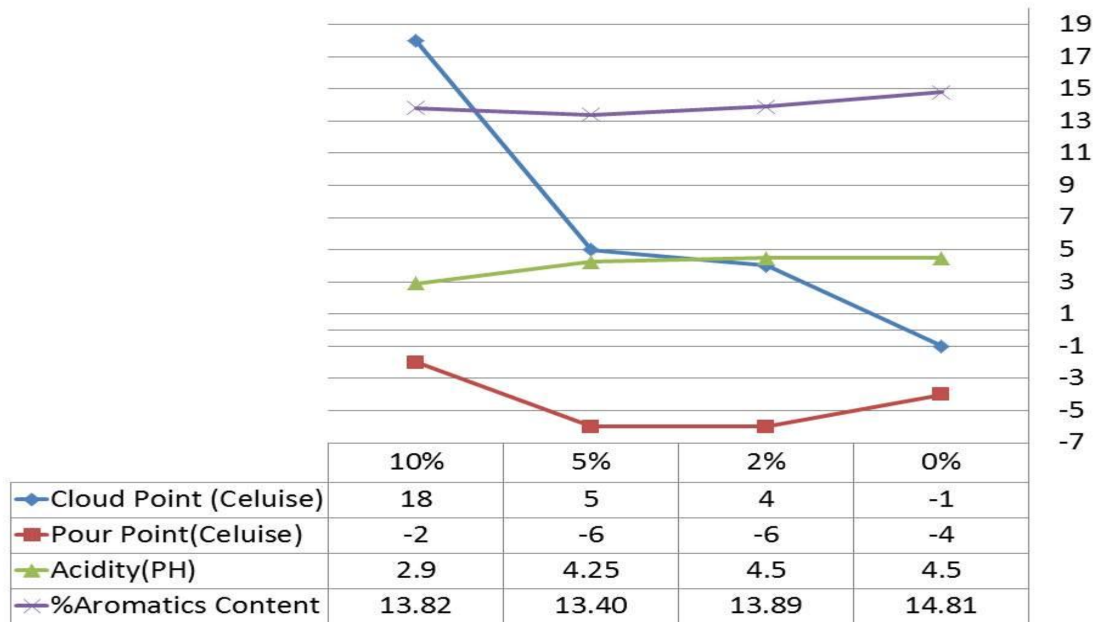
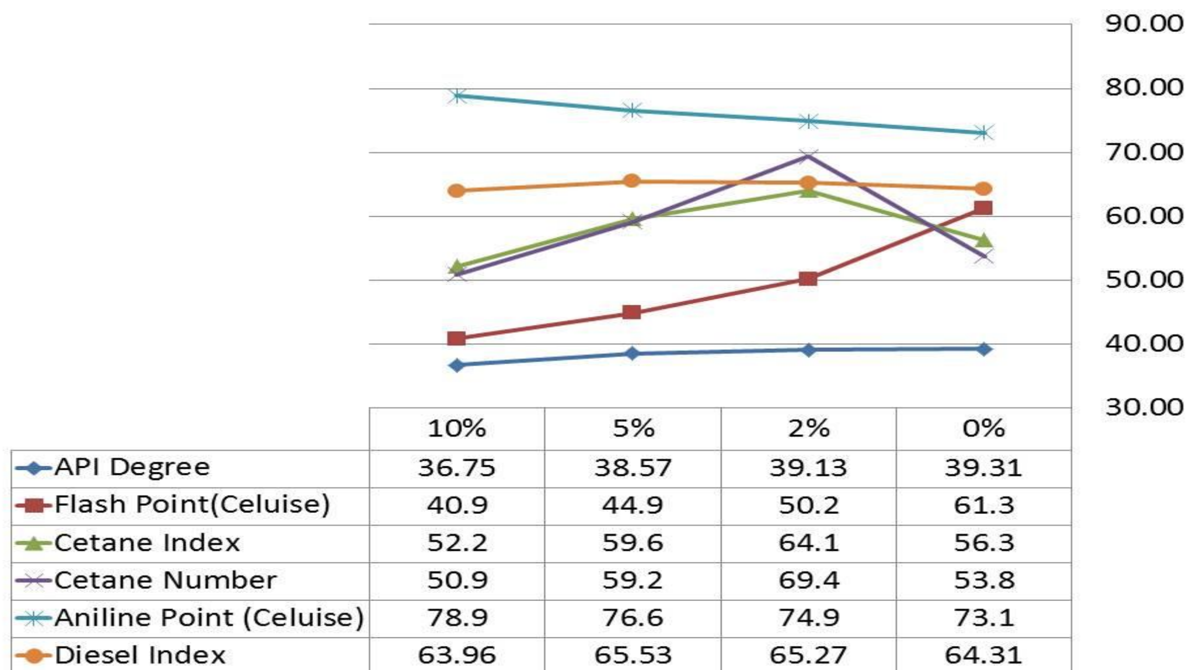


Figure (3): Diesel fuels properties vs. %vol. of GAA.



	10%	5%	2%	0%
API Degree	36.75	38.57	39.13	39.31
Flash Point(Celuise)	40.9	44.9	50.2	61.3
Cetane Index	52.2	59.6	64.1	56.3
Cetane Number	50.9	59.2	69.4	53.8
Aniline Point (Celuise)	78.9	76.6	74.9	73.1
Diesel Index	63.96	65.53	65.27	64.31

Figure-4-: Diesel fuels properties vs. %vol. of GAA.

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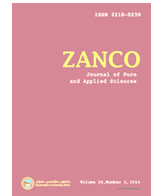

Effect of Sowing Dates and Two Chickpea Cultivars on Some Growth Parameters and Yield

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A B S T R A C T

A field study was conducted at Grdarasha Research Station of the College of Agriculture/ Salahaddin University– Erbil, during the growing season of 2017, to study the effect of sowing date on some growth parameters and yield of chickpea. A factorial experiment based on randomized complete block design (RCBD), with three replicates was used, sowing dates (January 20, February 9, March 1, and March 21), and two cultivars of chickpea (Rafidain and Gab) were implemented. Sowing at January, 20 produced the highest plant height (43.0 cm), leaf area (797.83 cm^2), leaf area index (2.66), dry matter (38.06 g m^{-2}), crop growth rate (7.90 g m^{-2} day^{-1}), number of primary branches $plant^{-1}$ (3.55), number of pod $plant^{-1}$ (21.60) and seed yield (2.160 t ha^{-1}). On the contrary sowing at March, 21 recorded the lowest of all above studied characteristics, also Gab cultivar surpassed the Rafidain cultivar in all characteristics studied except no. of seeds $plant^{-1}$ and 100-seed weight. From interaction sowing date with cultivars recorded the highest of all characteristics studied at sowing date January, 20 with Gab cultivars as well as Rafidain cultivar recorded the lowest of all studied characteristics at sowing date March, 21. There was positive highly significant correlation(r= 0.869) between seed yield and their components; no. of pods plant-1, no. of seeds pod-1 and 100-seed weight (r= 0.869, r= 0,810 and r= 0.706) respectively.

1. INTRODUCTION

Chickpea (*Cicer arietinum* L.) belongs to the family fabaceae, an annual and one of important pulls crop. Chickpea is an important source of protein for millions of people in the developing countries, in addition to having high protein content (20-22%), rich in fiber and minerals. Chickpea the fourth largest grain legume crops in the world, with a total production of 10.9 million tons from an area of 12.0 million with a productivity of 0.91 ha^{-1}. Major producing countries include India, Pakistan and Iran (FAO, 2016). Chickpea being a leguminous crop improves soil fertility by fixing atmospheric nitrogen up to 99 kg/ ha in available from (NH_3 and NH_4) in the root through the phenomena of symbiosis (Schwenke *et al.,* 1998). Among the various agronomic practices, sowing time is the single most important factor influencing the yield of chickpea. Optimum sowing time of chickpea may vary from one variety to another and also from one region to another due to variation of agro-ecological conditions. Different sowing

dates subject the vegetative and reproductive stages of the plant to various temperature, solar radiation and day length (Yadav *et al.,* 1999).The exposure of crop to low temperatures during germination and seedling establishment and to high temperature during flowering and seed formation phases under delay-sown chickpea results in drastic reduction in yield (Turner *et al.,*2001). Yield loss in chickpea can vary between 30% and 60% depending on genotype, sowing time, location, and climatic conditions during sowing season. Some chickpea genotypes have capacity to tolerate drought and in that case sowing time can be delayed. However, earlier or late sowing caused drastic reduction in yield and net profit compared with timely sowing (Dixit *et al.,* 1993). The different genotype growth response varies to different environment and their relative ranking usually differ (Eberhort and Russel, 1966) and ultimately decides the selection of genotypes for a particular or different sowing dates for stabilized higher yields (Perkins and Jinks, 1968). The chickpea genotypes differ in their yielding ability, this calls for a need to generate more information on the response of chickpea genotypes to the dates of sowing for greater yields in a given agro-climatic conditions. Hence, there is a need to identify appropriate sowing time, suitable variety and their interaction with respect to growth, development and yield performance. Yadav *et al*. (1999) reported that vegetative growth of forty chickpea genotypes belonging to the kabuli and desi types, continued into the reproductive stage for longer under normal than late sowing. Mahse et al. (2006) at Rahuri (Maharashtra) found that late sowing of chickpea resulted into 36.6% reduction in number of pod plant^{-1}. Virk *et al*. (2005) found that chickpea sown on November, 5 resulted in significantly higher seed yield (2039 kg ha^{-1}) followed by sowing on October, 25 (1837 kg ha^{-1}) and November, 25 (1826 kg ha^{-1}). Fallah (2008) at Khorram-Abad (Iran) showed that delay in sowing from March, 6 to April, 5 significantly reduced the number of grains pod^{-1}. Malik *et al*. (1995) reported that the two chickpea cultivars sown at the end of September and end of October gave mean seed yield of 972 and 836 kg ha^{-1}, respectively. Reddy and Ahlawat (1998) reported that desi variety BG 261 recorded significantly higher number of pods plant-1 than kabuli variety ICCC-32. Stieller et al. (1994) reported from New Zealand that the highest seed yield among desi chickpea cultivars was produced by ICCV-93801 (26.8 g plant-1) and among kabuli chickpea cultivars by ICCV-92338 (20.3 g plant^{-1}). Yadav *et al*. (1998) in New Delhi reported that delayed sowing beyond November decreased seed yield in both desi and kabuli chickpea. However reduction in kabuli chickpea was on the higher side than desi chickpea. Ku March et al. (2013) in Hisar (Haryana) reported that chickpea cv. HK 98-155 produced higher seed yield as compared to cvs. HK-1and HK 00-290.The present study was carried out to investigate some growth parameters and yield of two cultivars at different sowing dates.

2. MATERIALS AND METHODS

A field study was conducted at Grdarasha Research Station of the College of Agriculture/ Salahaddin University-Erbil (Latitude 36° 4' N and Longitude 44°2' E) 415 meters above sea level having annual rainfall (250-600 mm) during the season of 2017 to study the sowing date on some growth parameters and yield of two chickpea cultivars. A factorial experiment based on randomized complete block design (RCBD), with three replicates was applied. The first factor represents four sowing dates (January 20, February 9, March 1, and March 21), and the second factor represents two cultivars

of chickpea (Rafidain and Gab). Representative soil samples were taken from various locations of the field at depth of (0-30 cm) after tillage. These samples were air dried then sieved by using 2 mm sieve, then packed for analysis.

Experimental unit size was (2m×2 m) consisting of 5 rows of 180 cm length and 30cm a part. Sowing was done manually, two seeds were placed in each hole of 10 cm a part and sowing depth of 3 cm. Five plants were selected randomly from each experimental unit to study the plant height (cm), leaf area (LA)(cm2) and leaf area index (LAI): was calculated by viticanopy program application, Dry matter (gm^{-2}): Which represent the dry mass of total green parts of plant after drying at 80°C for (48-52) hours, then weight was converted to g m-2.Crop growth rate (CGR) g m^{-2} day^{-1}: It was calculated by dividing dry matter yield (gm^{-2}) at flowering stage by number of days from sowing to the flowering stage.

$$LAI = \frac{\text{Plant total leaf area per plant}}{\text{Average land area occupied by plant}}$$

$$\text{Crop Growth Rate (CGR)} = \frac{1}{GA} \times \frac{W_2 - W_1}{T_2 - T_1}$$

Number of primary branches $plant^{-1}$, number of pods $plant^{-1}$, number of seeds pod^{-1} and the 100-seed weight (g) were calculated. All middle-line plants of each experimental unit were harvested to calculated yield (kg ha^{-1}). The data was analyzed statistically for all studied traits according to analyses of variance using the Statistical Analysis System (SAS Institute, 2005). Duncan's multiple range test (DMR) at 5% level was used to the determine among means (Steel and Torrie, 1997). Simple correlation coefficient was calculated between the seed yield and other traits, and among the traits themselves.

Table (1): Metrological record for Grdarasha field during the rainfall season of (2017).

Parameter Year 2017	Air Temperature in (°C)		Monthly total rainfalls (mm)	Relative Humidity (R.H%)
January	**Maximum**	**Minimum**		
1-8	13.49	2.40	21.1	63.7
9-16	14.10	2.78		
17-24	14.77	3.77		
25-31	10.33	2.99		
February				
1-7	8.75	-0.35	6.4	54.7
8-15	14.66	5.61		
16-23	11.89	1.29		
24-28	19.19	5.72		
March				
1-8	21.02	11.37	28.8	50.6
9-16	19.83	9.72		
17-24	20.29	10.09		
25-31	23.62	9.93		
April				
1-8	19.45	4.55	11.7	42.4
9-16	23.52	12.13		
17-24	26.50	13.04		
25-30	29.27	15.37		
May				
1-8	30.72	17.40	3.3	24.8
9-16	35.48	19.72		
17-24	33.63	19.69		
25-31	36.64	19.85		
June				
1-8	37.42	21.44	0.0	17.0
9-16	39.97	23.82		
17-24	37.77	23.40		
25-31	43.95	26.09		

3. RESULTS AND DISCUSSION

Plant height (cm)

The data presented in table (2) confirms the existence of significant differences among all the factors studied the highest plant height (43.00 cm) when planted at January, 1 compared with sowing date on March, 21 that recorded (28.16 cm) because of the variations of temperature degrees at different sowing dates (Table 1). Among different varieties, plant height was also differed significantly up to maturity, plant height was recorded (37.75 cm) in Gab cultivar but Rafidain cultivar recorded (34.25 cm). However the interaction between sowing date and cultivars has significantly, the highest plant height (44.33 cm) when planted Gab cultivar at January, 1 compared with sowing date at March, 21 with Rafidain cultivar (27.33 cm). This result is in agreement with Mahse *et al.* (2006) in Rahuri (Maharashtra) noticed that plant height was decreased by 14.5% under late sown condition as compared to normal sown condition. Also, Chaitanya and Chandrika (2006) observed that chickpea cultivars sown on November, 1 exhibited significantly greater plant height (32.9 cm) as compared to November, 15, and December, 1 sown crops.

Leaf area (cm^2)

The data pertaining to leaf area as influenced by sowing dates varieties and their interaction (Table 2). Among different sowing dates January, 20 exhibited significantly higher leaf area (797.66 cm^2) as compared to March, 21 this increase in early sowing (January, 20) was due to the suitable temperature degrees for vegetative growth, hence increase in leaf area (Table 1). Among the different cultivars, Gab exhibited significantly higher leave area followed by Rafidain cultivars. The highest leaf area (851.33 cm^2) for Gab cultivar when planted at January, 1 compared with Rafidain cultivar when planted at March, 21 with that recorded lowest (522.33 cm^2). This result is in agreement with Mansur *et al.* (2010) observed that sown on first October exhibited significantly greater leaf area as compared to November, 15.

Leaf area index (LAI)

The results shown in table (2) shows significant differences of leaf area index among sowing dates, cultivars and their interaction the heights leaf area index was recorded at sowing date January, 20 (2.66) but the lowest has recorded in sowing on March, 21 (1.66). Among the different cultivars, Gab has significantly showed higher leaf area index followed by Rafidain cultivars. The highest leaf area index Gab cultivar (2.48) when was planted at January, 20 compared with Rafidain cultivar planted on March, 21 that recorded the lowest (1.48). This result is in agreement with of Haloi and Baldev (1986) who reported that leaf area index gradually decreased with delay in sowing of chickpea crop.

Dry matter ($g\ m^{-2}$)

The results of the analysis of variance in the table (2) showed significant differences for all the studied factors, plants cultivated at January, 20 produced higher yields of dry matter (38.80g m^{-2}), while the plants cultivated on March, 21 gave the dry matter (12.27g m^{-2}) due to rise in temperature degrees at late sowing dates (Table 1) which resulted in decrease in leaf area and plant height (Table 2) and then lowering in dry matter for these dates, the highest dry matter has recorded at January, 20 sowing date with Gab cultivar (38.47g m^{-2}) as compared with sowing on March, 21 with Rafidain cultivar (12.48g m^{-2}). This result is in agreement with the results of Mansur *et al.* (2010) who observed that chickpea cultivars sown on first October exhibited significantly greater dry matter as compared to November, 15.

Crop Growth Rate (g m^{-2} day^{-1})

The results of analysis of variance in table (2) showed significant differences among all studied factors and their interaction .The cultivated plants at January, 20 gave the highest rate of crop growth reached (7.9g m^{-2} day^{-1}), while on March, 21 gave the lowest rate for this trait (5.23 g m^{-2} day^{-1}). Almost the plant cultivated at January, 20 with Gab cultivar gave highest crop growth rate (8.33g m^{-2} day^{-1}), while Rafidain cultivar produced the lowest for this trait (5.06 g m^{-2} day^{-1}) when planted onMarch,21. From the data of table (4), it was noticed that there was a high significant correlation relationship between crop growth rate with leave area reached (r = 0.987).

No. of primary branches $plant^{-1}$

Table (3) shows the effect of sowing date, cultivars and their interaction on this trait components analysis of chickpea. The maximum among the sowing dates was January, 20 which was one of four sowing dates; whereas the minimum was for March, 1 and March, 21which was (2.95 and 2.88), respectively. However, the different cultivars, Gab cultivar obtained significantly higher no. of primary branches followed by Rafidain cultivar. The highest was on January, 20 (3.66) with Gab cultivar, but the least value was recorded for Rafidain cultivar on March, 21 (2.70). Also Rehman *et al.*(2015) showed number of branches per plant at October, 15 was significantly higher over other sowing dates and cultivars had non-significant number of branches per plant.

No. of pods $plant^{-1}$

The results of no. of $podsplant^{-1}$are displayed in table (3). A wide variation was observed between no. of pods/ $plant^{-1}$, the highest number of no. of pods $plant^{-1}$ was on January, 20 which was (21.60), while the lowest number was recorded on March, 21 which was (6.06). The maximum was for Gab cultivar (18.22); whereas the minimum was for Rafidain cultivar (14.38). Referring back to table (3), there was coincidence in the results of Gab cultivar at January, 20which also give highest number (23.32), but the lowest was (7.88) from Rafidain cultivars at March, 21.The obtained results in the study indicated that the response of chickpea cultivars to sowing times were different. Therefore, to determine suitable sowing date in chickpea different cultivars should be studied (Kaya*et al.,* 2010).

No. of seeds pod^{-1}

The of no. of seeds $pod^{-1}$are displayed in table (3) shows that the highest was recorded for the sample collected on January, 20 (1.60), which was near to the sample on February, 9 (1.08), followed by (1.07) at March, 1 which all other samples were similar statistically. Whereas the minimum was on March, 21 (0.56). However the cultivars that the optimum results for Gab cultivar (1.13). Considering the interaction between sowing date and cultivars, the highest was (1.27) for Gab at January, 20 but the lowest value was recorded from Rafidain cultivar at March, 21 (1.00). However, delay in sowing resulted in adverse effect of climate which, resulted in poor crop stand and short time to complete their life cycle, especially after November, 15 (O'Toole *et al.,*2001).

100-seed weight (g)

Table (3) shows100-seed weight the highest for the sample collected on January, 20 (26.44 g), which was similar to sample February, 9, March, 1, and March, 21 which were(26.40, 24.90, and 24.78 g) respectively that all samples were similar. The highest interaction were recorded for the sample collected from Gab at January, 20 (28.69 g), but the least was (21.01g) for Rafidain cultivar at March, 21. The lowest number of 100-seed

weight and seed yield in early and late autumn sowing dates were due to encounter of flowering and fertilization stages with high and low temperatures respectively (Chaitanya *et al.,*2006).

Seed yield (kg ha^{-1})

Seed yield displayed in table (3) shows that the highest at January, 20 (2159.55kg ha^{-1}), while the lowest were at March, 1 (1579.94kg ha^{-1}). The highest cultivars were also for Gab (1725.50 kg ha^{-1}) but the lowest value was recorded for Rafidain (1590.75 kg ha^{-1}) the increase of yield on January, 20 led to increase in all yield components for this sowing date (Table 3). These variations in numbers, in the samples confirm that the interaction between sowing date and cultivars are different. The optimum value was on January, 20 for Gab (2274.36kg ha^{-1}), whereas the minimum was on March, 21 for Rafidain (959.33kg ha^{-1}). The results are in general agreement with the published results that in 2012-13 growing season, sowing date and cultivars interaction significantly influenced the chickpea seed yield per hectare (Rehman *et al.,*2015). The result showed that different sowing date and chickpea cultivars had individually significant effect on yield and yield component (Yucel *et al.,* 2008). From the data of table (4) it was noticed that there was a highly significant correlation relationship between seed yield with no. of primary branches per plant^{-1}, no. of pods per plant, no. of seeds per pod and 100-seed weight which were (r = 0.882, r =0.869, r =0.810, and 0.706) respectively.

Table 2: Effect of sowing date on some growth parameters of two chickpea cultivars.

Sowing date		Plant height (cm)	Leaf area (cm^2)	Leaf area index	Dry matter (g/m^2)	Crop Growth Rate (g/m^2/day)
January, 20		43.0 a	797.83 a	2.66 a	38.06 a	7.90 a
February, 9		38.16 ab	673.66 b	2.24 b	30.09 ab	6.78 b
March, 1		36.16 b	593.83 c	1.98 c	24.63 b	5.95 c
March, 21		28.16 c	499.33 b	1.66 d	12.47 c	5.32 d
Cultivars		**Plant height (cm)**	**Leaf area (cm^2)**	**Leaf area index**	**Dry matter (g/m^2)**	**Crop Growth Rate (g/m^2/day)**
Rafidain		34.25 b	595.8 b	2.01 ab	25.4 b	6.16 ab
Gab		37.75 a	676.6 a	2.25 a	27.6 a	6.82 a
Sowing date × Cultivars		**Plant height (cm)**	**Leaf area (cm^2)**	**Leaf area index**	**Dry matter (g/m^2)**	**Crop Growth Rate (g/m^2/day)**
January, 20	Rafidain	41.66 ab	744.33 b	2.48 b	38.01 a	7.48 b
	Gab	44.33 a	851.33 a	2.84 a	38.12 a	8.33 a
February, 9	Rafidain	34.66 bcd	640.33 d	2.13 d	28.24 abc	6.51 d
	Gab	41.66 ab	707.00 c	2.35 c	33.55 ab	7.22 c
March, 1	Rafidain	35.00 abc	522.33 e	1.87 e	22.52 bc	5.76 f
	Gab	37.00 abc	626.00 d	2.08 d	26.74 bcd	6.15 e
March, 21	Rafidain	27.33 d	476.66 g	1.58 g	15.48 cd	5.06 g
	Gab	29.00 cd	522.33 f	1.74 f	12.48 d	5.58 f

Table 3: Effect of sowing date on yield a yield component of two chickpea cultivars.

Sowing date		No. of primary branches/ plant	No .of pods/plant	No. of seeds/pod	100 seed weight (g)	seed yield (kg/ha)
January, 20		3.55 a	21.60 a	1.60 a	26.44	2159.55 a
February, 9		3.16 ab	18.88 ab	1.08 a	26.40	1898.68 b
March, 1		2.95 b	12.27 b	1.07 a	24.90	1579.94 c
March, 21		2.88 b	6.06 c	0.56 c	24.78	991.17 d
Cultivars		**No. of primary branches**	**No. of pods/plant**	**No. of seeds/pod**	**100 seed weight (g)**	**seed yield (kg/ha)**
Rafidain		3.00 ab	14.38 b	1.08 ab	25.14	1590.75 b
Gab		3.20 a	18.22 a	1.13 a	26.11	1725.50 a
Sowing date × Cultivars		**No. of primary branches**	**No. of pods/plant**	**No. of seeds/pod**	**100 seed weight (g)**	**seed yield (kg/ha)**
January, 20	Rafidain	3.22 ab	19.88 ab	1.19 a	28.65 a	2044.73 b
	Gab	3.66 a	23.32 a	1.27 a	28.69 a	2274.36 a
February, 9	Rafidain	3.16 b	17.11 bc	1.07 ab	26.70 ab	1845.00 d
	Gab	3.21 ab	19.8 ab	1.12 a	27.59 abc	1952.37 c
March, 1	Rafidain	2.88 b	12.66 c	1.05 b	24.22 bcd	1515.33 f
	Gab	3.11 ab	17.88 bc	1.09 ab	25.21 abc	1644.48 e
March, 21	Rafidain	2.70 b	7.88 e	1.00 b	21.01 b	959.33 h
	Gab	2.85 b	11.88 d	1.02 b	22.86 bc	1023.00 g

Table 4: Correlation coefficient analysis among the traits of chickpea cultivars

	Plant height	Leaf area	Leaf area index	Dry matter	Crop Growth Rate	No. of primary branches/ plant	No. of pods/plant	No. of seeds/pod	100-seed weight	Seed Yield
Plant height	1.000									
Leaf area	0.785 **	1.000								
Leaf area index	0.786 **	1.000 **	1.000							
Dry matter	0.827 **	0.779 **	0.779 **	1.000						
Crop Growth Rate	0.780 **	0.987 **	0.987 **	0.745 **	1.000					
No. of primary branches/plant	0.567**	0.518 *	0.518 *	0.804 **	0.360 *	1.000				
No .of pods/plant	0.768 **	0.795 **	0.794 **	0.919 **	0.791 **	0.826 **	1.000			
No. of seeds/pod	0.631 **	0.524 **	0.658 **	0.820 **	0.605 **	0.301 *	0.788 **	1.000		
100-seed weight	0. 222	0.951 **	0.135	0.438 *	0.137	0.021	0.144	0.026	1.00 **	
Seed Yield	0.792 **	0.951 **	0.950 **	0.820 **	0.938 **	0.882 **	0.869 **	0.810 **	0.706 **	1.00 **

*and ** significant at level 0.05 and 0.01 respectively

4. CONCLUSIONS

It is concluded that early sowing date on January, 20 excelled the other sowing date (February 9, March 1, and March 21) for all characters studied, also Gab cultivar surpassed Rafidain cultivar for all characteristics except 100-seed weight, the highest seed yield (2.160 t ha^{-1}) and other yield components were recorded at sowing date January, 20, while the lowest were at March, 1 (1.580 t ha^{-1}). From interactions the highest seed yield (2.274 t ha^{-1}) recorded from Gab cultivar with sowing date at January, 20, while the minimum was recorded for Rafidain cultivar at March, 21 (0.959 t ha^{-1}).

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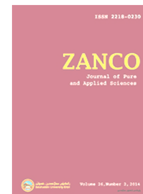

The Physico-Chemistry and Benthic Macro-Invertebrate Diversity and Abundance of Nwaniba River, Akwa Ibom State, Nigeria

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A B S T R A C T

The physico-chemical parameters and benthic macro-invertebrates' diversity and abundance of Nwaniba River in Akwa Ibom State were studied between October, 2014 and January, 2015. Surface water samples and benthic macro-invertebrate samples were collected from six (6) sampling stations according to standard methods. The mean values of the measured water parameters were: Temperature (28.31±0.41) ^{o}C, Dissolved Oxygen (3.74±0.17) mg/l, Conductivity (16.41±1.80) μs/cm, Salinity (0.028±0.001) ppt, Total Dissolved Solids (15.75±4.53) mg/l and pH (6.9±0.12). A total of 7 macro-invertebrate species belonging to 3 classes were encountered in the following order of abundance**:** Insecta (55.0%),Mollusca (40.0%) and Annelida (5.0%). The most abundant species were *Chironomus spp* and *Neritina cristata* accounting for 8 (40.0%) and 5 (25%) respectively. Whereas *Segmentorbis augustus*, *Neritina rubricata*, *Pachymelania byronensis* and *Tubifex tubifex* were the least abundant with 1 (5%) organism each. The low abundance of benthic macro-invertebrates species and the total absence of the EPT indexed taxa, relative to a higher abundance of a pollution indexed taxa (*Chironomus spp*) may directly indicate the pollution stress level of this Nwaniba River system. Hence, there is need for pollution management of this river.

1. INTRODUCTION

It is needless to emphasize on the importance of water in our daily lives, as water used for different purposes has its own requirements for its physico-chemical composition and purity. The physical and chemical characteristics of water are often used as measures for determining its quality and purpose. The functioning of an aquatic ecosystem and its ability to support life forms depends to a great extent, on its physical, chemical and biological characteristics (Bunn *et al.,* 1999).

The consideration of water quality is important in wetland studies because of a host of interacting physical, chemical and biological factors which may influence the trophic structure, biomass (Hellawell, 1986), and consequently its suitability for the distribution and diversity of indigenous organisms (Swingle, 1967). According to Sala *et al.* (2000), biodiversity loss and its effects are predicted to be much greater for aquatic than terrestrial ecosystem, thus wetland ecosystems ought to be analyzed on regular basis to

confirm their ability to support different aquatic life forms.

In Nigeria, water availability and quality are deteriorating due to climate change and land use activities e.g. petroleum exploration, logging, sewage discharges etc and these activities impacts greatly on the water quality thus introducing hazardous substances that may suppress indigenous populations and diversity of aquatic organisms (UNEP, 1991; Boyer and Grue, 1995). Aquatic organisms offer valuable information regarding their environmental conditions, thus they can be used as tools when evaluating the physical, chemical and biological impacts of their surrounding (Karr and Chu, 1999). This is the reason, benthic macro-invertebrates are important and integral parts of any aquatic ecosystem as they tend to form the basis of the trophic level, and their diversity and abundance greatly depends on the physical and chemical conditions of the substratum (Sharma and Chowdhary, 2011; Chatzinikolaou *et al.,* 2006). Therefore, they may be considered good indicators of water quality because of their susceptibility to various environmental disturbances and tolerance level.

The various human activities in and around Nwaniba River may have some level of impact on the river ecosystem process (such as sedimentation) and the ecology of its indigenous species resulting from both point and non-point pollution sources. Impact of such disturbances on various aquatic species or non-living elements (i.e water quality) may have long term consequences (Edokpayi *et al.,* 2000). Hence, this study aims at using the abundance and diversity of the benthic macro invertebrates of Nwaniba River to assess its health status and exposure to pressures due to pollution stress.

2. MATERIALS AND METHODS

2.1. Study area

Nwaniba River (Figure 1) lies between latitude $5^0 2'51''$N and longitude $8^0 2'41''$E. This location experiences a mean annual rainfall, mean temperature and relative humidity of about 2500mm, 32°C and 75% respectively. Vegetations such as *Pennisetum purpurem, Nypha frutican, Nymphaea lotus* and other tropical hydrophytes were found in the study area. The river is used for artisanal fishing, transportation and domestic purposes.

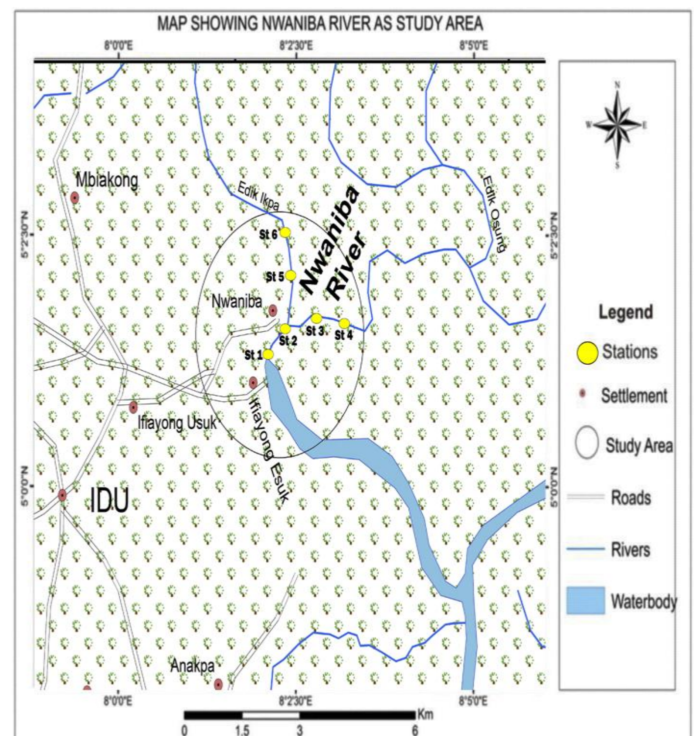

Fig. 1: Map of Nwaniba River showing sampling stations (1-6)

2.2. Measurement of water variables

Surface water parameters were determined fortnightly between October 2014 and January 2015 between the hour of 7:00am and 9:00am. In-situ measurement of water quality parameters were carried out at the study site using appropriate devices withstandard

methods (APHA, 1998). The Dissolved Oxygen concentrations were determined using a DO meter (model Harmam DO meter) and the results expressed as mg/l. The pH was measured using an electronic pH meter (Oakton waterproof instruments phlestr10). Water temperatures were measured in (^{o}C) using a digital thermometer embedded in an Extect meter model Extik EC400 which was also used to measure Salinity, Conductivity and TDS respectively.

2.3. Collection of benthic macro-invertebrate samples

Benthic macro-invertebrate samples were collected with a Van-Veen grab. The sediment was poured into polyethylene bags, labeled and taken to the laboratory for analysis. The sampled sediments were poured into a sieve with 0.5mm mesh size for washing, as described by Holme and Mc-Intyre (1984) and George *et al*. (2009). The residue retained in the sieve was poured into a clean tray for sorting. The sorted macro-benthos was identified, using the guides of Macan (1959) and Edmunds (1978). The numbers were counted and recorded.

2.4. Data Analysis

Water quality and biological data for benthic macro invertebrates were analyzed using quantitative indices to determine central tendency of the measured water quality parameters, the relative abundance and diversity of species and groups, correlation analysis and Canonical correspondence analysis (CCA) using PAST and SPSS softwares versions 3.0 and 17 respectively.

3. RESULTS AND DISCUSSION

3.1. Physico-chemical parameters:

Table 1 shows the spatial variations of Range, Mean and Standard error of physico-chemical parameters of Nwaniba River measured during the study period.

Although the water quality parameters of Nwaniba River were within recommended limits as stipulated by NESREA for tropical aquatic lives, the observed spatial variations of water quality parameters of this river system could be attributed to riparian vegetation cover, and various human activities around the sampling stations.

3.2. Macro benthic invertebrate composition and abundance

A total of 7 species of macro benthic invertebrates belonging to three taxa were encountered (Table 2). The most abundant taxa were Arthropoda with a percentage composition of 55%, while the least was Annelida with percentage composition of 5%. The *Chironomus spp.* in the class Insecta was the most diverse recording the highest abundance, while *Neritina rubricata, Pachymelania bryonensis, Segmentorbis angustus, Tubifex tubifex* recorded the least abundance.

3.3 Diversity indices

High values of Shannon-Wiener Index_**H** were recorded for phylum Arthropoda (1.30) and a value for Annelida (0). Higher values for species Dominance_**D** were recorded for phylum Annelida (1.0) and the least value of (0.29) for Arthropoda. Evenness ranged between (0.84) for Mollusca and (1) for Annelida (Table 3).

The benthic macro-invertebrate diversity, distribution and abundance were relatively low. Diversity indices values considered as a strong bio-indicator of ecosystem health (Gaskill, 2004), may have revealed the health status of

this river system (Table 3). According to Yakub and Ugwumba (2009), bigger lotic water bodies tend to have low macro invertebrate richness. Major human activities (like saw milling) may have affected the nutrient level of this river, thus compromising the sedimentation structure of the river system.

Both increased nutrient levels and sedimentation are known elements of pollution and they have negative impacts on macro invertebrate abundance (Aura *et al.,* 2010). Harding *et al.* (1999) suggested that high periphyton biomass due to nutrient enrichment and sedimentation, may favor richness of species of Chironomids, Mollusks and Annelids at the expense of EPT indexed taxa, a suggestion which is similar to the observations in the present studies.

3.4. Correlation Analysis

The following Correlation existed between the measured water quality parameters and benthic species encountered. Water parameters such as pH and TDS showed positive relationships with Mollusca at $P<0.05$ (Table 4).

3.5. The relationship between Physico-chemical variables and plankton abundance

The Canonical correspondence analysis (CCA) ordination diagram was employed to express the relationships existing between benthic biota and the water variables (Figure 2).

A higher abundance of *Chironomus spp*. in this river system could be attributed to: their possession of haemogloblin - a pigment that transports and stores dissolved oxygen (Tyokumbor, 2002); a well-known fact which helps this insect to adapt and thrive in polluted water environment. The positive correlation that existed between Mollusca abundance with pH and TDS suggested that they require water with high TDS and pH and these could be attributed to their mode of nutrition. Result of the CCA revealed that Insect group had much preference to Station 3, with TDS as the most influencing factor.

Table 1: The Spatial variations, Range, Mean and Standard error of water quality parameters at studied sites

Water Parameters	Spatial Variations						Range	Mean
	Station 1	Station 2	Station 3	Station 4	Station 5	Station 6		± Std. Err.
Temperature (˚C)	28.7	28.2	28.3	28.1	28.3	28.2	**25.5-31.7**	**28.31 ±0.41**
Dissolved oxygen (mg/L)	3.6	3.8	3.7	4.0	3.8	3.5	**2.1-5.4**	**3.74 ±0.17**
Conductivity (μ/cm)	17.2	17.9	26.2	16.4	16.9	15.5	**14.1-58.6**	**16.41 ±1.8**
Salinity (ppt)	0.029	0.028	0.027	0.029	0.028	0.028	**0.01-0.09**	**0.028 ±0.01**
pH	6.9	6.8	6.9	6.9	7.1	6.8	**6.3-8.2**	**6.9 ±0.12**
Total Dissolved solids (mg/L)	9.4	8.2	15.5	8.6	23.0	30.6	**6.3-99.4**	**15.75 ±4.5**

Table 2: Benthic Macro Invertebrate Diversity, Distribution and Abundance at studied sites

Benthic Macro Invertebrate	Spatial Distribution						Total	Percentage Abundance	
	Station 1	**Station2**	**Station 3**	**Station 4**	**Station5**	**Station6**			
Mollusca									
Neritina afra (Sowerby, 1836)	-	3	-	1	1	-	5	25%	**40 %**
Neritina rubricate (Sowerby, 1836)	-	1	-	-	-	-	1	5%	
Pachymelania bryonensis (Wood, 1828)	-	-	-	1	-	-	1	5%	
Segmentorbis angustus (Jickeli, 1874)	-	-	1	-	-	-	1	5%	
Arthropoda									
Chironomus sp. (Meigen, 1803)	-	-	2	3	1	2	8	40%	**55 %**
Ceratopogonid sp.	-	-	2	-	-	1	3	15%	
Annelida									
Tubifex tubifex (Muller, 1774)	-	-	-	-	1	-	1	5%	**5 %**
TOTAL	0	4	5	5	3	3	20		

Table 3: Diversity index of benthic macro invertebrate group at studied sites

Diversity index	**Macro invertebrate Taxa**		
	Annelida	**Arthropoda**	**Mollusca**
Dominance_D	1	0.29	0.34
Shannon_H	0	1.30	1.21
Evenness_e^H/s	1	0.91	0.84

Table 4: Pearson Correlation between water quality parameters and benthic macro invertebrates (P< 0.05)

	Temp. (^{0}C)	**DO (mg/l)**	**pH**	**Salinity (ppt)**	**Conductivity (µS/cm)**	**TDS (mg/l)**
Annelida	0.017	0.272	-0.162	-0.119	-0.110	0.010
Arthropoda	0.000	0.162	0.176	0.078	-0.152	-0.96
Mollusca	-0.223	-0.375	**0.474***	-0.123	0.072	**0.503***

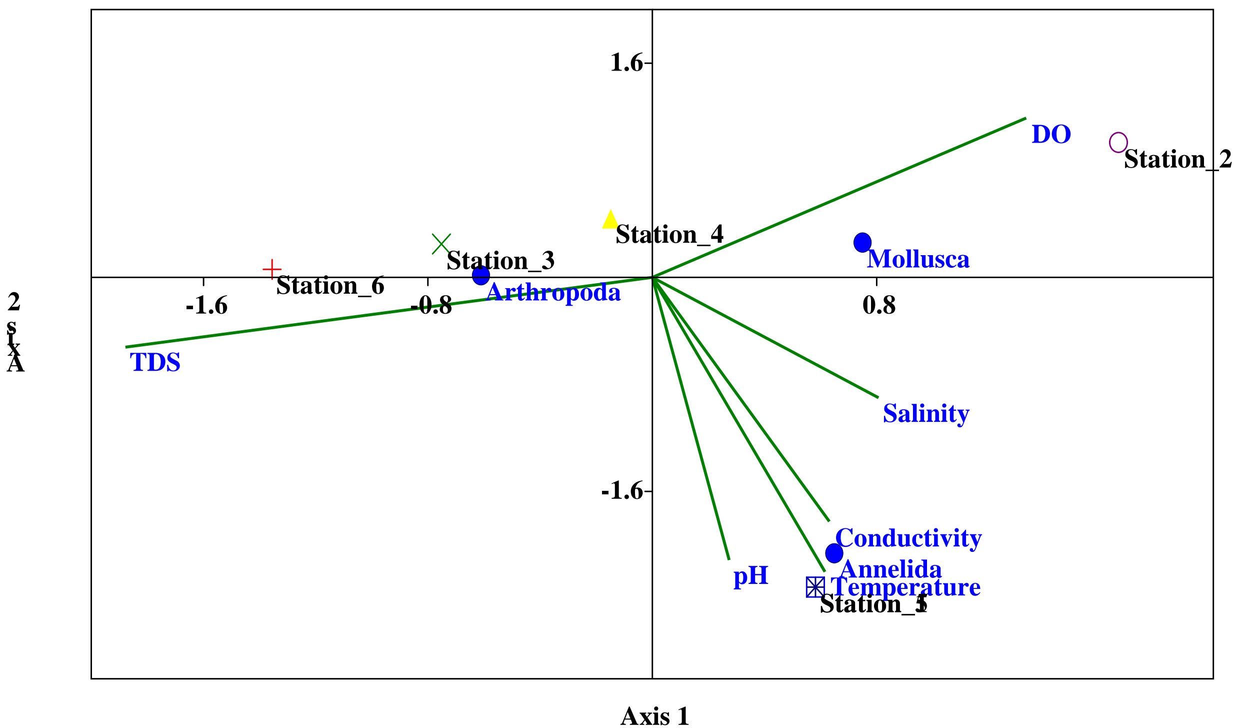

Fig. 2: Canonical correspondence analysis (CCA) triplot ordination diagram with 3 benthic macro-invertebrate groups, 6 quantitative variables and studied stations 1 to 6

4. CONCLUSION

The water quality parameters of Nwaniba River may still fall within recommended limits as stipulated by NESREA for tropical aquatic organisms, however, the sporadic distribution and the lower benthic macro invertebrates diversity and abundance (with a higher abundance of the pollution-tolerant taxa; *Chironomus spp.*) serves as biological measure reflecting that Nwaniba River could be under stress of organic pollution from anthropogenic sources such as saw dust, human feaces, detergents etc. Hence, need for strict pollution management of this natural resource.

Conflict of Interest

There is no conflict of interest.

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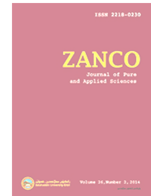

Association between Anemia during Pregnancy and post partum hemorrhage and perinatal outcome among women with vaginal Births in slemani maternity teaching hospital

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ABSTRACT

Over more than half a million women die due to complications of pregnancy and childbirth each year. In the developing countries the risk of dying in childbirth is 175 times that of developed countries.

Postpartum hemorrhage, is the leading cause of maternal death in developing countries characterized by bleeding (>500 mL) after birth of the baby.

Aim of the study: To evaluate whether maternal anemia contributes to greater blood loss at childbirth and 24 hours postpartum.

Patients and methods: This study is a cross sectional descriptive study, the target 233 patients will be pregnant ladies in labour seen in labour ward in slemani maternity teaching hospital, throughout the period of January first 2017 to August first 2017, evaluated the state of anemia in her third trimester, the hemoglobin level was measured for all the studied women, then they were divided in to groups according to their HB level.

Results: The median age of studied women was 28.5years, and the median gestational age duration was 38.8 weeks, 44.6 percent were hemoglobin level was less than 11 gram / dl, 20.6 percent of these patients developed post partum hemorrhage. 6.4 percent need blood transfusions, 28.9 percent need medical treatment. Commonest causes of post partum hemorrhage among these patients were inertia, genital injury respectively.

Conclusions: The finding of this study support the association between anemia at delivery and the potential risk of PPH which remains currently debated.

1. INTRODUCTION

Anemia in pregnancy is defined as hemoglobin level below 11 g/dl (WHO)(Frass, 2015). It is one of the public health problems mostlyin developing countries(Organization, 2007).World Health Organization (WHO) reported that the prevalence of anemia during pregnancy indeveloping countries exceeds 50% (Frass, 2015).

A normochromic, normocytic anemia may occur fromthe 7–8th week of gestation, due to the physiologicalincrease in plasma volume that is relatively greater thanthe increase in red cell mass. However, the hemoglobin (Hb) should not fall to <11 g/dl in the first, or <10 g/dlin the second and third trimesters(Haram et al., 2001, Milman et al., 2000). More marked anemia may be due to iron, folate, or more rarely, vitaminB12 deficiency or hemoglobinopathy(Clark et al., 2012).

In pregnancy, anemia is mainly nutritional due to dietary deficiency of iron and folates(Sanghvi et al., 2010) .but impaired absorption, chronic blood loss, increased requirement, concurrent medical disorders and

malaria are other contributingfactors for anemia(Kalaivani, 2009). It has long been considered thatanemia increases the risk of postpartum hemorrhage (PPH)(Ramanathan and Arulkumaran, 2006) and the two conditions together contribute to 40–43% of maternal deaths in Africa and Asia(Christian, 2008).

The effect of iron deficiency (before anemia) on maternal and fetal well-being is not fully understood, but mild deficiency is linked to increased delivery bleeding, poor fetal iron stores and an increased placenta: fetus weight ratio(Hindmarsh et al., 2000). Severe maternal iron deficiency is associated with premature delivery and low birth weight(Haram et al., 2001).

Hemorrhage is still one of the leading causes of maternal mortality in the United Kingdom with postpartum hemorrhage playing a significant role in the deaths of nine women in the last triennial report(Oyelese and Ananth, 2010).

Primary postpartum hemorrhage is defined as the loss of 500 mL of blood from the genital tract following, but within the first 24 hours of, the delivery of the baby(Waterstone et al., 2001).

A caveat is added in that if the blood loss is <500 mL, but is sufficient to cause hypovolaemic shock in the patient, this is also classified as a primary postpartum hemorrhage. A new definition of massive postpartum hemorrhage has been introduced, being the loss of greater than 1000, 1500 or 2500 mL of blood. Owing to the relatively low risks of blood loss below these levels, the incidence of this complication is being used in some units as an indicator of the standard of maternity care and is being promoted by the Royal College of Obstetricians and Gynecologists as one of their clinicalindicators on the maternity scorecard(Arulkumaran et al., 2008).

Despite the global significance of postpartum hemorrhage, little is known about factors thatcontribute to postpartum hemorrhage, especiallyin less-developed areas where 99% of maternaldeaths occur. Severe anemia may weaken uterinemuscular strength or lower resistance to infectiousdiseases, contributing to postpartum hemorrhageand subsequent maternal mortality(Rush, 2000, Bergström, 2003).

2. MATERIALS AND METHODS

Study design: This study was cross sectional descriptive study.

Sample size and duration of the study: The study included 233 pregnant women admitted to the labor ward of the maternity teaching hospital in slemani city, throughout the period of January first 2017 to August first 2017, evaluated the state of anemia in her third trimester or sending for hemoglobin during the admission and these pregnant ladies will followed up during the period of labor and post-delivery for the first 24 hours for postpartum hemorrhage and perinatal outcome. Data collection was performed by the self-administered method. Intrapartum and post-partum blood loss was measured by specific container labeled in milliliter, calculate and weighing the number of pads had been used during labor and post-delivery.

Inclusion criteria: Single tone term pregnancy.

Exclusion criteria: twin and higher order multiple pregnancy, patients with past history of post partum hemorrhage, patients with medical disease , history of bleeding tendency, patients on anti-platelets drugs (aspirin, LMWH).

Descriptive statistics and analytical statistics were used to test statistical difference and associations using SPSS version 21.

Statistical analysis: The data was entered in to Excel sheet, then transported into Statistical Package for the Social Sciences (SPSS version 21.0) software program for statistical analysis. Were calculated for all variables, as well as analytical statistics were conducted to find the relations between variables; by using Chisquare, and analysis of variance (ANOVA). A p-value < 0.05 was considered as significant

3. RESULTS

The mean age of patients which participative in our study was 28.5years, and the mean gestational age duration was 38.8 weeks, as shown in table 1

Table 1. Selected characteristics of patients enrolls in the study

Variables	Range	Minimum	Maximum	Mean	SD
Maternal age (Years)	28	15	43	28.5	6.5
Maternal weight (kg)	34	56	90	72.6	8.6
Gravida	7	1	8	2.9	2.0
Para	6	1	7	2.1	1.5
Gestational age in (weeks)	7	34	41	38.8	1.3
Durations of delivery (hours)	10	2	12	6.9	2.8
APGAR	4	6	10	8.5	0.8
Fetal weight (Kg)	2.7	2.3	5.0	3.3	0.4

The mean maternal weight was 72.6 kg, most of the patients had more than 2 babies as a mean, the mean durations of delivery were near 7 hours. As shown in table 2 the characteristics variable of the study groups.

Table 2: Effects of potassium and magnesium on RBC, HG, HCT, MCV and MHC in 2K1C hypertensive rats

Variables	Frequencies	Percentages
Employment		
Yes	80	34.3
No	153	65.7
Mother		
education	29	12.4
illiterate	81	34.8
Primary	50	21.5
Secondary	73	31.3
school		
University		
Hb (gm)		
>11gm	105	45.1
9.5-11gm	104	44.6
8gm-9.5gm	11	4.7
<8gm	13	5.6
Mode of		
delivery	133	57.1
NVD	67	28.8
IOL	33	14.2
Augmentation		
OL		
Postpartum		
bleeding	185	79.4
< 500ml	32	13.7
500ml-1000ml	16	6.9
>1000ml		
Blood		
transfusions	217	93.6
No	16	6.4
Yes		
medical Rx		
Yes	67	28.9
No	165	71.1
Causes PPH		
inertia	8	88.9
genital injury	1	11.1
Gender		
Female	144	61.8
Male	89	38.2

As shown the results in table 3 the patients with hemoglobin level more than 11 gram had no blood loss more than 1000 ml during follow up period compared with 6.8% of patients with hemoglobin less than 11 gram developed post partum blood loss more than 1000 ml which was statistically significant compared with no patients developed post partum hemorrhage with hemoglobin more than 11 gram.

50 percent of patients (8 patients) with blood loss more than 1000 ml need blood transfusion compare with only 1.6 percent (3 patients) need

blood transfusion among patient with blood loss less than 500ml which was statistically significant.

The mode of delivery either spontaneous vaginal delivery, augmentations of labor, inductions of labor does not affect the amount of post partum hemorrhage with the p value 0.045 which was statistically not significant.

Table 3 shows the relation between post partum hemorrhage and the variable of the study groups,

variables of the study group				
Variables	**Postpartum hemorrhage**			**P values**
	< 500ml N (%)	**500ml-1000ml N (%)**	**>1000 ml N (%)**	
Hb (gm)				<0.001
>11gm	89 (48.1%)	16 (50%)	0 (0%)	
9.5-11gm	96 (51.9)	5 (15.6)	3 (18.8)	
8gm-9.5gm	0 (0%)	6 (18.8)	5 (31.3)	
<8gm	0 (0%)	5 (15.6)	8 (50.0)	
Mode of delivery				0.045
NVD	100(54.1 %)	19 (59.4%)	14 (87.5%)	
IOL	56 (30.3%)	11 (34.4%)	0 (0%)	
Augmentation OL	29 (15.7%)	2 (6.3%)	2 (12.5%)	
Blood transfusions				<0.001
No	182 (98.4%)	27 (81.3%)	8 (50.0%)	
Yes	3 (1.6%)	5 (12.5%)	8 (50.0%)	

medical Rx				
Yes	19 (10.3%)	32 (100%)	16 (100%)	<0.001
No	165 (89.7%)	0 (0%)	0 (0%)	

The result in table 4. Shows only 3 patients with hemoglobin more than 11 gram need blood transfusion after delivery compare with 13 patients with hemoglobin less than 11 grams need blood transfusion which was statistically significant.

5 patients with severe anemia (Hb< 8 gm) develops uterine atony (inertia) ,in which 2 of them need hysterectomy because of uncontrolled bleeding compare with only 1 patient develops uterine atony who had hemoglobin more than 11 gram.

The perinatal outcome, regarding Apgar score as shown in table 5 was 8.6±0.9 Mean±S.D in groups of patients with hemoglobin more than 11 gram compare with Apgar score of 7.8±0.6 Mean±S.D in groups of patients with hemoglobin less than 8 gram which was statistically not significant.

Table 4. The relation between the severity of anemia and the variable of the study group

Variables	Anemia severity (Hb)				P values
	>11gm	9.5-11gm	8gm-9.5gm	<8gm	
Employment					
Yes	69 (65.7%)	5 (4.8%)	0 (0%)	6 (46.2%)	<0.001
No	36 (34.3%)	99 (95.2%)	11 (100%)	7 (53.8%)	
Mother education					
Illiterate	4 (3.8%)	15 (14.4%)	6 (54.5%)	4 (30.8%)	
Primary	6 (5.7%)	71 (68.3%)	0 (0%)	4 (30.8%)	<0.001
Secondary school	31 (29.5%)	14 (13.5%)	3 (27.3%)	2 (15.4%)	
University	64 (61%)	4 (3.8%)	2 (18.2%)	3 (23.1%)	
Mode of delivery					
NVD	56 (53.3%)	62 (59.6%)	6 (54.5%)	9 (69.2%)	<0.001
IOL	22 (21.0%)	39 (37.5%)	4 (36.4%)	2 (15.4%)	
Augmentation OL	27 (25.7%)	3 (2.9%)	1 (9.1%)	2 (15.4%)	
Blood transfusions					
No	102 (97.1%)	104 (100%)	11 (90.9%)	0 (0%)	<0.001
Yes	3 (2.9%)	0 (0%)	2 (9.1%)	11 (84.6%)	
Causes PPH					
inertia	1 (100%)	1 (100%)	1 (100%)	5 (83.3%)	0.905
genital injury	0 (0.0%)	0 (0%)	0 (0%)	1 (16.7%)	

Table 5. Shows the Mean±S.D of severity of anemia and the relation to the variables.

Table 5. The association between the variables and Mean±S.D of anemia severity

Variables	Anemia severity (Hb)				P values
	>11gm Mean±S.D normal	9.5-11gm Mean±S.D mild	8gm-9.5gm Mean±S.D moderate	<8gm Mean±S.D severe	
Maternal age (Years)	28.4±5.7	26.9±6.2	34.2±5.4	35.8±6.9	<0.001
Maternal weight (kg)	72.9±9.9	71.2±6.6	80.9±5.3	80.4±7.6	<0.001
Gravida	2.3±1.4	2.9±2.1	4.8±2.3	5.4±1.6	<0.001
Para	1.5±0.9	2.4±1.4	4.3±1.9	4.2±1.7	<0.001
Gestational age (Weeks)	38.6±1.4	38.9±1.2	39.3±0.7	39.4±1.1	0.066
Durations of delivery (hours)	7.7±2.6	6.4±2.9	6.1±2.2	5.5±2.6	0.002
APGAR	8.6±0.9	8.5±0.8	8.5±0.9	7.8±0.6	0.025
Fetal weight (Kg)	3.2±0.3	3.3±0.3	4±0.7	3.7±0.4	<0.001

4. DISCUSSION

Our finding in this study of 233 pregnant lady underwent vaginal delivery in labor ward in Slemani maternity teaching hospital is a strong association between moderate-to-severe anemia atlabor and greater severityofblood loss at delivery and postpartum.

The patients with hemoglobin level more than 11 gram had no blood loss more than 1000 ml during follow up period compared with 16 patients with hemoglobin less than 11 gram developed post partum blood loss more than 1000 ml which was statistically significant, our results agree with Justine A. Kavle et al. at 2008 which Enroll 158 Zanzibar Pregnant Ladies at labor, they found strong association between anemia and post partum hemorrhage(Khan et al., 1997).

Severe anemia is hypothesized to impair tolerance of postpartum hemorrhage and contribute to maternal death, possibly due to the failure of women to endure such excessive blood losses and late arrival at admission(Allen, 2000, Alauddin, 1986).

In this study the mode of delivery either spontaneous vaginal delivery, augmentations of

labor, inductions of labor does not affect the amount of post partum hemorrhage with the p value 0.045 which was statistically not significant,

Which agree with Claudio G. Sosa et al. at 2010,in this study which done on Latin-American population showsaugmentation and/or induction of labor were not associated with increased risk of postpartum hemorrhage(Bais et al., 2004, Xiong et al., 1994, Ohkuchi et al., 2003).

Our study disagrees with Geelhoed D et. al. 2006 how done studies among African populationsreported no association between maternal anemia and postpartum hemorrhage inamong Ghanaian women(Geelhoed et al., 2006).

In this study 5 patients with severe anemia (Hb< 8 gm) develops uterine atony (inertia) ,in which 2 of them need hysterectomy because of uncontrolled bleeding compare with only 1 patient develops uterine atony who had hemoglobin more than 11 gram, which agree with Kaima A. Frass et al. study done in Al Thawra General hospital. Fifty-three cases were included in the study. Results: Most of the hysterectomies women 80.75% (17/21) had Hb levels ⩽ 7 versus 12.5% of the non-hysterectomies patients.

5. CONCLUSIONS

The finding of this study support the association between anemia at delivery and the potential risk of PPH which remains currently debated. Also we provide evidence of the association between severe anemia and severe uterine atony requiring emergency hysterectomy.

6. RECOMMENDATION

Further studies with larger sample size to confirm these findings are required. In this group of anemic women who develop severe PPH due to uterine atony, early decision of hysterectomy to save their lives is potential and should be considered when other measures are ineffective.

Further study requires confirming associations between other risk factor for post partum hemorrhage in non-anemic pregnant lady at labor.

The result of our study highlights the need to increase the population awareness to utilize the available maternity care services along with the promotion of iron and folates supplementation for all pregnant women. The screening and therefore treatment of anemia must be essential part of antenatal care components particularly in setting where malaria and other infectious diseases are prevalent, the specific cause of anemia was not considered in this study.

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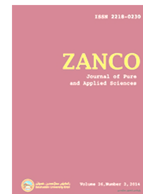

The Impact of Flow rate on Inhaler Dose Delivery from a Dry Powder Inhaler using a Two Stage Impinger

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A R T I C L E I N F O

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A B S T R A C T

The purpose of this study is to investigate the effect of various flow rates and morphology of spray-dried transferosomes depending on the amount of drug delivered in "respirable" fractions from a monodose dry powder inhaler (DPI). A two-stage impinger (TSI) was used to investigate the deposition of salbutamol sulphate (SS) as a model drug incorporated within four different carrier-based transferosomes (sorbitol, inulin, trehalose and maltodextrin). The amount of drug was collected from each stage of the TSI, as well as the device and capsule at three different flow rates: 30, 60 and 90 L/min, and subsequently quantified by high performance liquid chromatography (HPLC). Results from this study showed that the 30L/min flow rate was not as effective as 60L/min or 90L/min at delivering drug to the lower stage of the TSI. However, 90L/min flow rate had a higher drug deposition in the lower impinger when compared to 60L/min. Sorbitol was found to be the least effective carrier at delivering drug to the lower chamber of the TSI, followed by maltodextrin, whereas trehalose and inulin were more spherical and smaller in size and were found to be the most effective carrier systems in the spray-dried formulations. In conclusion, trehalose and inulin-based transferosomes were the most effective delivery system for depositing the drug into the lower part of two stage impinger.

1. INTRODUCTION

The respiratory system can be exploited as a route for non-invasive and patient-friendly drug delivery (Shakshuki and Agu, 2017). Pulmonary drug delivery has gained much importance in recent decades due to the ability of targeting the drug directly to the lung tissues both for local and systemic effects (d'Angelo et al., 2015; Newman, 2017; Kaur, 2017).

Air flow rate is a patient variable which is considered to be important because it can affect the amount of drug delivered from the device to the peripheral airways (Buttini et al., 2015). Inter-individual variation occurs between healthy people, and further variation in air flow rate occurs in people with compromised lung function (Muralidharan et al., 2015; Chen et al., 2016). Two of the very common diseases of the lung are asthma and chronic obstruction

pulmonary disease (COPD). Both of these are inflammatory; asthma is characterised by narrowing of the airways and COPD by the destruction of the alveolar walls (Nakawah et al., 2013). There are many drugs used in these conditions, for example, salbutamol sulphate which is a selective ß2-adrenergic receptor agonist which relieves asthma by dilating the bronchioles. These drugs are administered by inhalation using metered dose inhalers, dry powder inhalers or nebulisers (Elhissi et al., 2007). Dry powder inhalers are a breath controlled drug delivery systems in which the patient's own flow rate is required to achieve sufficient release of the drug from DPI devices and subsequent diffusion of the drug into the lungs, thus the inspiratory flow rate of the patient has a significant influence on delivery of drugs prepared as DPI formulations (Yokoyama et al., 2007). Pulmonary drug delivery is advantageous as local targeting allows minute drug dosing because drug metabolism in the lung is lower than the gastrointestinal tract and the liver with less systemic side effects (Paranjpe and Müller-Goymann, 2014). However, there are also disadvantages of pulmonary delivery; the duration of action of the inhaled drug is often short-lived because of the mucocilliary clearance and metabolism by the enzymes and this necessitate frequent dosing (Shah et al., 2012).

There are three main mechanisms of particle deposition in the airway, inertial impaction, sedimentation and Brownian diffusion. Inertial impaction occurs in the upper airway which is caused by the velocity and the mass of the particle. Sedimentation occurs in the peripheral airway and Brownian diffusion is relevant to particles < 1 μm (Heyder, 2004; Tsuda et al., 2013).

In this study, the drug was incorporated within transferosomal dispersions containing high carbohydrate concentrations, followed by spray drying to yield flowable transferosomal powders. Sorbitol, inulin, trehalose or maltodextrin was used as the stabilizing carriers in the process of spray drying. The delivery performance and characteristics of the delivered formulations were studied and the influence of type of carrier and flow rate through the two stags impinger was evaluated.

Spray drying is a process which provides control over particle size and morphology; reduce powder density (Yang et al., 2015; Omer et al., 2018). Moreover, agglomerated particles generated via spray drying may have high flowability (Augsburger and Hoag, 2008). These characteristics using spray dried powders can promote the ability of inhaled particles to reach the lower respiratory airways, bearing in mind that particles should have a size less than 5μm to be regarded "respirable" or in "fine particle fraction" (Rojanarat et al., 2012). Other factors that may influence the respirability of powdered formulations include the specific features and design of inhaler device (Ibrahim et al., 2015; Berkenfeld et al., 2015).

It is important to remember that even healthy individuals have different inhalation profiles, leading to different deposition patterns in the respiratory tract. Diseases such as asthma and COPD are additional contributors to different patterns of deposition following drug inhalation. In a study of inhalation flow measured through a variety of DPI devices, it was found that patients with COPD generate an air flow rate between 32 and 98 L/min and patients with asthma generate an air flow rate of 45 to 110 L/min (Chrystyn and Price, 2009). When patients are given inhalation therapy for their condition, they are all given the same dose regardless of their inspiratory flow rate. It was reported that 28 of 233 patients who inhaled corticosteroids for asthma could not

achieve the optimal flow rate of 60 L/min, of whom 5 were treated for exacerbation of symptoms (Barnes et al., 1998). Thus, it is very important to clarify the relationship between inspiratory flow rate and amount of drug delivered into the lungs when using a DPI. Hence the aim of this study was to investigate the impact of flow rate on the respirable dose, and the effect of higher flow rates on oropharyngeal impaction and also to compare the effect of different carriers (sorbitol, inulin, trehalose and maltodextrin) based transferosomes.

2. MATERIALS AND METHODS

2.1. Materials

Sorbitol, trehalose, and methanol (HPLC grade 99.9%) were purchased from Sigma-Aldrich, UK. Water (HPLC grade) and absolute ethanol were purchased from Fisher scientific, UK. Inulin and maltodextrin were purchased from VWR, UK. Salbutamol sulphate (99%), sodium 1- hexane sulfonates monohydrate (99%) and glacial acetic acid (99%) was all supplied by Alfa-Aesar, UK. Soya phosphatidylcholine (SPC, Lipoid S-100) was a gift from Lipoid, Switzerland.

2.2. Methods

2.2.1 Preparation of spray-dried transfersomes

The transfersomes were formulated by thin film hydration method (Ghanbarzadeh and Arami, 2013; Ali et al., 2015; Hassanpour Aghdam et al., 2016). Briefly, desired amounts of SPC (100 mg), Salbutamol sulphate (5 mg) and span 80 (15 mg) were dissolved in 20 ml of ethanol. The container tightly closed, protected from light and maintained at room temperature make sure of formation of complete and homogeneous solution. The mixture was transformed to a round-bottomed flask for solvent removal using a rotary evaporator (Heidolph, Germany) at reduced pressure at 40 °C for two hours to remove traces of solvent. The dried film was hydrated with deionised water including the carbohydrate carriers (sorbitol, inulin, trehalose or maltodextrin) in 1:5 w/w lipids to carrier ratio. Transfersomes were spray-dried using the Buchi mini spray dryer B-290 (Buchi, Switzerland). The inlet temperature was set up at 130°C and atomizer pressure was 800 KPa, the feed rate was adjusted to 13%, and the outlet temperature was 76 ± 1°C. The transfersome powder was transferred from the collecting chamber into a desiccator until used.

2.2.2 Scanning electron microscopy (SEM)

Surface morphology of the transferosome particles was examined by SEM. A sample was sprinkled onto an aluminum stub and coated with gold by a sputtering technique using a JFC-1200 Fine Coater (JEOL, Tokyo, Japan) for 120 s. The particles were observed under SEM (Quanta-200, FEI) at 20 kV.

2.2.3 HPLC analysis

A buffer comprising sodium hexane sulfonate (5mM) in water was mixed with methanol (75:25 v/v) to prepare the mobile phase to which glacial acetic acid was added to constitute 1% of the total volume. The HPLC instrument (Agilent 1200 with UV detector; Hewlett-Packard Co., USA) was set up with a symmetry C18 column (150 mm_4.6 mm, 5 m; Waters Ltd, UK) and samples were analysed at 276 nm. The mobile phase flow rate was adjusted to 1mL/min at 40 oC, and the volume of automatically injected sample set to 20 μL

(Elhissi et al., 2006). A calibration curve of ascending drug concentrations was constructed.

2.2.4 In vitro powder aerosolisation

Powder aerosolisation performance and particle deposition profile were determined in vitro using a TSI (figure 1) (Copley Scientific Limited, Nottingham, UK; British Pharmacopoeia, 2000) with the Miat Monodose inhaler device (Miat S.p.A., Milan, Italy) as the aerosol dispersion device. The flow rate was adjusted to 30, 60 or 90 L/min using the critical flow controller (TPK 2000) and a flow meter (DFM 2000) (Copley Scientific Limited, Nottingham, UK). Spray dried formulation (25 mg) was weighed and transferred into size 3 HPMC (Hydroxypropyl methylcellulose) capsules (Qualicaps Europe, Madrid, Spain), which were individually installed in the inhaler device. The inhaler was attached to the “mouth” of the TSI following its assembly using deionised water as collection medium (7 and 30 ml in upper and lower stages respectively). Each capsule was actuated from the inhaler over 5 s for each measurement. The amount of powder deposited in the different stages of the impinger was determined using HPLC (section 2.2.3).

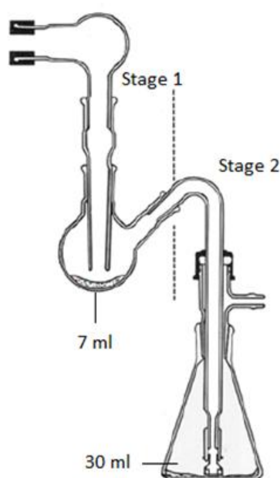

Figure (1): Schematic representation of a two stage impinger (Hallworth et al., 1987)

3. Statistical analysis

All experiments were performed in triplicates and values were expressed as mean ± standard deviations. Statistical significance was assessed using one way analysis of variance (ANOVA) and student t-tests, as appropriate. Values with $P < 0.05$ indicate that the difference is statistically significant.

4. RESULTS AND DISCUSSION

The morphologies of spray-dried transferosome particles were primarily dependent on the type of carrier and the differences in morphologies were clearly evident (figure 2). SEM shows that inulin and trehalose-based transferosomes (figure 2b,c) were small spherical particles with low tendency for aggregation. While, sorbitol-based transferosomes (figure 2a) showed particles of various size with irregular shape particles and greater tendency for aggregation compared to inulin and trehalose based transferosomes. Furthermore, the discrete particles were smaller, spherical and smooth when maltodextrin was used as carrier, the spray-dried particles formed massive agglomerates, owing to the hygroscopic nature of maltodextrin as reported by previous studies (Loret et al., 2004; Sansone et al., 2011; Akhilesh et al., 2012).

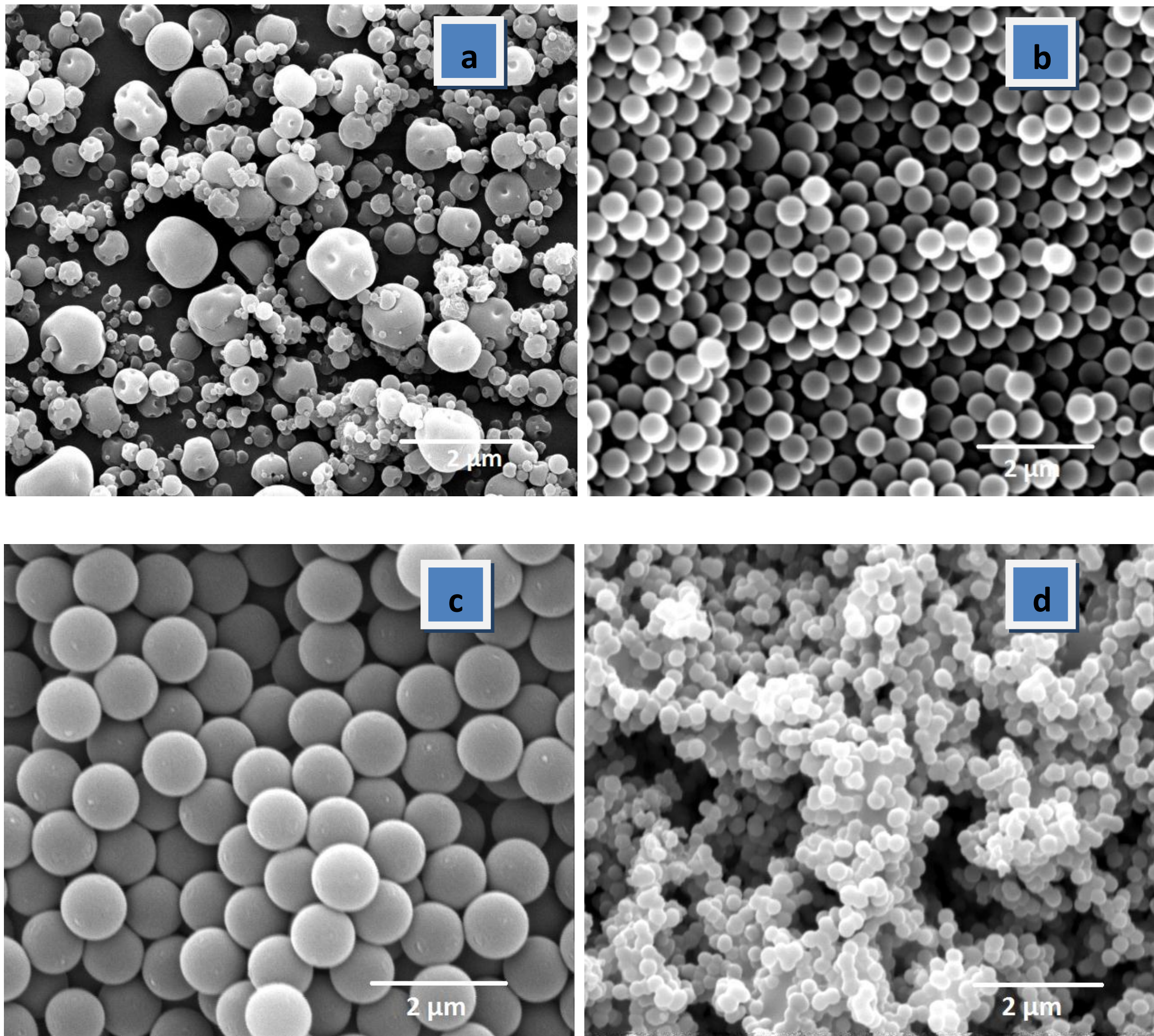

Figure (2): SEM images of spray-dried transferosomes, (a) Sorbitol-based transferosomes (b) Inulin-based transferosomes (c) Trehalose-based transferosomes (d) Maltodextrin-based transferosomes.

Figure 3, 4 and 5 shows the effect of flow rate on the amount of drug delivered to both upper and lower compartments of the TSI, as well as that left within the device and capsule. The results demonstrated that with increased flow rate, there were fewer drugs left in the device and capsule and more drugs delivered to the lower chamber of the TSI. These results agree with Borgström et al., 1994 and Cheng, 2014, they studied that inspiratory flow rate has an important effect on the drug deposition at various stages of impinger. When looking at each individual tansferosome formulation and the percentage of drug delivered to the lower chamber of TSI. At 30 L/min maltodextrin and sorbitol-based transferosomes (figure 3) had the greatest percentage of drug deposition when compared to inulin and trehalose-based transferosomes.This difference could be attributed to the surface morphology of different transferosomes (Figure 1). Sorbitol-based transferosomes (figure 3) were shown to have the least amount of drug left in the device ($p=0.0002$) and capsule ($p<0.0001$), therefore

more of the drug was delivered to the TSI. The transferosomes which show the highest percentage of drug left in the capsule and device were inulin-based transferosomes (45.21±12.64%) and trehalose-based transferosomes (62.9±11.26), which may not be as beneficial to the patient in practice as they could result in a sub-therapeutic dose being delivered (Jones et al., 2014).

Results at 30 L/min showed that maltodextrin-based transferosomes were the most effective transferosome formulation to use for inhalation delivery of salbutamol sulphate because this formulation achieved the highest drug delivery to the lower chamber of the TSI ($p<0.0001$). The reason for this could be that the low flow rate resulted in a reduced velocity of the spray, with no enough force to overcome the cohesive forces between the sticky particles of inulin and trehalose-based transferosomes (Cheng, 2014). Therefore, most of the particles remained in the device and capsule and even after delivery deposited in the upper chamber of the TSI because the agglomerated particles were not small enough to reach the lower chamber of the TSI. Inhaled particles must have a diameter of 1.0–6.0 µm (Timsina et al., 1994; Saini et al., 2007), under 7 µm (Newman et al., 1994) or 0.5–8.0 µm (Davies et al., 1976) to be defined as ‘respirable particles’ and to be deposited on the bronchi or alveoli. Particles larger than ‘respirable sizes’ are generally deposited within the upper respiratory tract by inertial impaction and then expelled from the respiratory system (Martins et al., 2015). Maltodextrin-based transferosome particles were irregular in shape, as shown by SEM (figure 2c), and seemed less sticky on handling compared to inulin and trehalose-based transferosomes. They may not require the generated spray velocity to be as high as with the other formulations in order to overcome the cohesive forces between particles and deliver the drug to the lower chamber of the TSI.

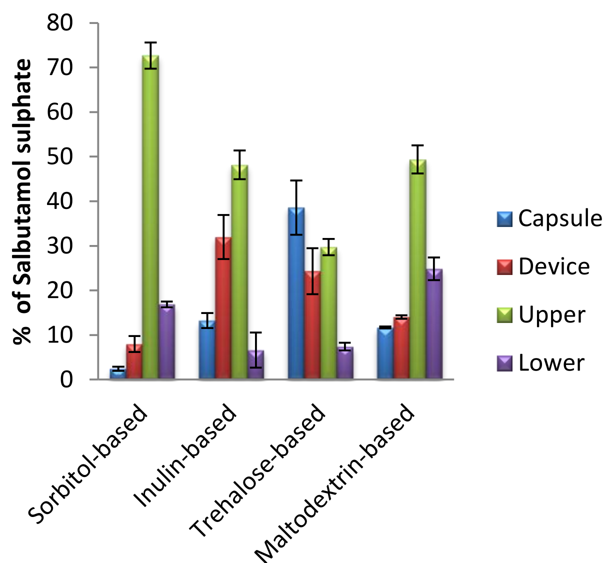

Figure (3): The percentage of drug deposition with four different carriers (Sorbitol, inulin, trehalose and maltodextrin) at flow rate of 30L/min.

At flow rate 60 L/min (figure 4), there was a noticeable decrease in the amount of drug left in the device ($p=0.23$) and capsule ($p=0.0007$) for all four transferosome formulations, compared to flow rate 30L/min. Therefore, there was an increase in the amount of drug delivered to the lower chambers of the TSI ($p<0.0001$). Borgström et al., 1994 have studied the lung deposition of budesonide and terbutaline sulphate at different inspiratory flow rates. They found that decreasing the inspiratory flow rate resulted in reducing the amount of drugs deposition in the lungs. When looking at different transferosome formulations (figure 4), sorbitol-based transferosomes were the least effective transferosomes to deliver drug to the lower chamber of the TSI, as drug delivery was 14.2±1.8% ($p<0.0001$). This was

low compared to inulin and trehalose-based transferosomes, which had more than a two-fold increase in the percentage of drug delivered to the lower chamber of the TSI (44.95±1.95 and 45.51±2.84%, respectively) ($p<0.0001$). Maltodextrin-based transferosomes achieved a higher drug delivery to the lower chamber of the TSI (31.53±0.25%) compared to sorbitol formulation ($p<0.0001$). They were less effective, however, compared to inulin and trehalose-based transferosomes ($p<0.0001$). The reason for this could be due to the velocity of the spray generated at 60L/min, which was sufficient to overcome the cohesive forces between small (<5 µm), spherical particles of inulin and trehalose-based transferosomes. These formulations have good flow properties at flow rate 60 L/min and can be delivered into the lower chamber of TSI. While sorbitol and maltodextrin-based transferosomes were not sticky as inulin and trehalose-based transferosomes but their particles seemed bigger once agglomerated, which meant that most of the particles were delivered from the device but did not reach the lower chamber of the TSI, and mostly deposited in the upper compartment of the TSI instead. Figure 4 shows that 60 L/min was the flow rate least likely to deliver drug to the upper chamber of the TSI, particularly with inulin and trehalose-based transferosomes, when compared to flow rate 30 and 90 L/min, which can be desirable. Maltodextrin-based transferosomes were shown to be less effective than inulin and trehalose formulations ($p<0.0001$) but more effective than sorbitol-based transferosomes ($p=0.0043$). The reason for this could be that at 30 L/min there was not enough velocity to force the drug particles to the lower chamber of the TSI.

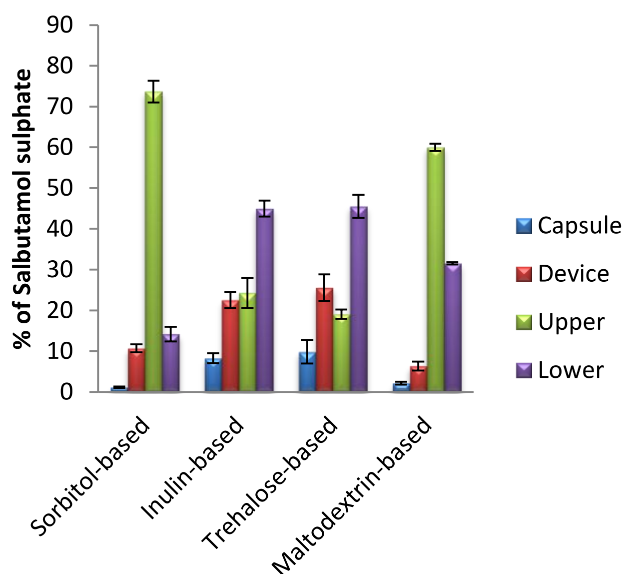

Figure (4): The percentage of drug deposition with four different carriers (Sorbitol, inulin, trehalose and maltodextrin) at flow rate of 60L/min.

At flow rate 90 L/min (figure 5), inulin and trehalose-based transferosomes clearly showed a higher percentage deposition of drug in the lower chamber of the TSI (57±2.89% and 55.42±4.31%, respectively) when compared to sorbitol and maltodextrin-based transferosomes (21.22±0.64 and 38.69±4.80, respectively) ($p<0.0001$). At flow rate 90 L/min, there was a significant decrease in the amount of drug left in the device ($p<0.0044$) and capsule ($p<0.0001$) when compared to flow rate 30 and 60 L/min for all formulations. Therefore, there was an increase in the amount of drug delivered to the lower chambers of the TSI, compared to flow rate 30 and 60 L/min ($p<0.0001$). This is probably because the high flow rate has helped with disaggregating the particles or overcome the cohesive forces between particles, resulting in allowed the maximum amount of drug to be delivered from the device (Prime et al., 1997). Also, at 90

L/min the velocity was too high, leading to inertial impaction on the oropharynx, therefore a reduced velocity is preferred to prevent the deposition of particles on the oropharynx by inertial impaction (Chrystyn, 2003; Tsuda et al., 2013).

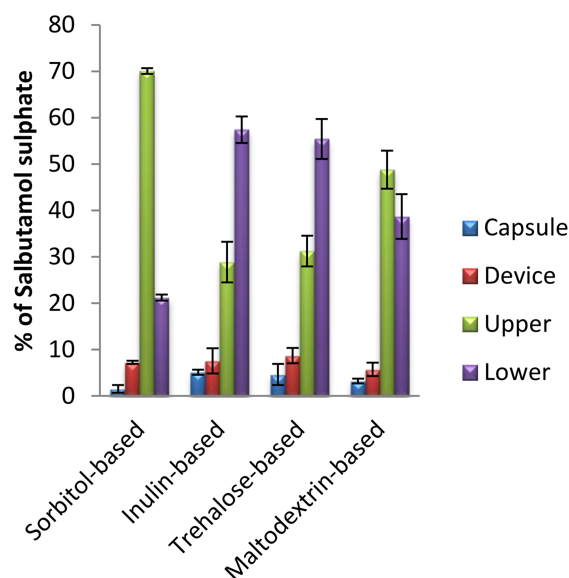

Figure 5: The percentage of drug deposition with four different carriers (Sorbitol, inulin, trehalose and maltodextrin) at flow rate of 90L/min.

5. CONCLUSIONS

This study investigated that 90L/min was the best flow rate compared to 30 and 60L/min at delivering drug to the lower chamber of the TSI. Flow rate 60L/min had an only slightly lower amount of drug in the lower chamber of the TSI, particularly with inulin and trehalose-based transferosomes. Results indicate that sorbitol formulations were the least effective transferosomes followed by maltodextrin, as they failed to deliver an adequate dose of drug to the lower chamber of the TSI, at flow rate 60 and 90L/min. These two formulations delivered a higher amount of SS to the lower chamber of the TSI at flow rate 30L/min, compared to inulin and trehalose-based formulations. This study proved that the inspiratory flow rates and the type of carbohydrate carrier have a significant role on the amount of drug delivering to the different positions of two-stage impinger.

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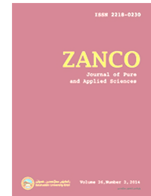

Detection of Biofilm Formation in *Pseudomonas aeruginosa* Isolates from Clinical Specimens

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A R T I C L E I N F O

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A B S T R A C T

The ability of a microorganism to develop biofilm is considered as a marker of clinically relevant infection. Infections caused by *P. aeruginosa* are difficult to treat as the majority of isolates exhibit high level of innate resistance to many antibiotics and tendency to form biofilms. The study was undertaken to investigate biofilm-forming capabilities of *P. aeruginosa* isolated from clinical specimens by two main different methods tissue culture plate (TCP) method, and congo red agar (CRA) method. Further, to investigate the antimicrobial resistance profile among biofilm producing isolates. A total of 300 specimens were collected from patients. Ninety six isolates of *P. aeruginosa* were obtained from various clinical samples. We applied Vitek-2 automated system as a panel of antimicrobial agents. TCP method and CRA method assay was chosen to detect the biofilm formation. Out of the 96 isolates, the results by TCP method and CRA method were 84(87.5%) and 76(79.1%) isolates respectively. TCP method was the most sensitive method for detection of biofilm production. TCP method detected 78 as strong, 6 as moderate and 12 as weak or non- biofilm producers. By CRA method, 65,11and 20 of isolates were strong, moderate and weak or non-biofilm producers, respectively. The antibiotic resistance pattern of *P. aeruginosa* was found higher in biofilm producers than in biofilm non-producers.TCP method was considered as the gold standard and reliable method for detection of biofilm formation. We conclude that there has been a positive association between drug resistance and biofilm formation of *P. aeruginosa*.

1. INTRODUCTION

Pseudomonas aeruginosa (*P.aeruginosa*) is a remarkably one of the most adaptive prevalent nosocomial pathogens. They have been implicated in serious and life-threatening infections (Bo Fu *et al.*, 2017).Infections caused by *P. aeruginosa* associated with a higher death rate particularly in clinical settings (Karthic and Gopinath, 2016). They are frequently responsible for various acute and chronic opportunistic infections (Heydari and Eftekhar, 2015). Biofilm formation is another important characteristic of *P.aeruginosa* contributes to the chronicity of infections as they reduce susceptibility to antimicrobial agents and consequently decreased therapeutic options (Bo Fu *et al.* , 2017).It is now commonly accepted that biofilm formation is the most common mode of growth of bacteria (Mathur *et al.*, 2006).Biofilm is defined as an assemblage of microbial cells that is associated (not removed by gentle rinsing) with a surface and covered by an exopolysaccharide matrix (slime) (Bose *et al.*, 2009, Stepanovic´*et al.*, 2007).Various changes occur during their transition from planktonic to a surface attached community. In response to certain environmental signals,

and exhibit an altered phenotype with respect to growth rate and gene transcription (Bose *et al*., 2009, Hassan *et al*., 2011).These phenotypic differences are manifested in various ways, depending on the species of bacteria. According to a publication by the National Institutes of Health, more than 60% of all infections are related to biofilms (Bose *et al*., 2009).Microorganisms growing in a biofilm are generally well protected against environmental stresses, antibiotics, desiccation, disinfectants as well as the host immune system and consequently are notoriously difficult to eradicate (Steenackers *et al*., 2012). Biofilm formation is main medical significance as they reduce the sensitivity to the antimicrobial agents. Further, the vicinity of cells within a biofilm can facilitate a plasmid exchange and promote the distribution of antimicrobial resistance (Niveditha *et al*., 2012).There are various number of tests are available to detect biofilm production. These methods include the TCP, TM, CRA, bioluminescent assay and light or fluorescence microscopic (Mathur *et al*., 2006).Nevertheless, the TCP (microtiter plate) method remains among the most frequently used assays for investigation of biofilm, and a number of modifications have been developed for the *in vitro* cultivation and quantification of bacterial biofilms (Stepanovic´ *et al*., 2007).Under laboratory conditions, this form of biofilm is characterized by the expression of cellulose and curli fimbriae, two major extracellular matrix components in *Salmonella* spp. and *E. coli* (Monteiro *et al*., 2011, Uhlich *et al*., 2006). The in vitro production of curli fimbriae and cellulose is usually manifested as a distinct colony morphotype on congo red agar plates. Both components are capable of interacting with the dye Congo Red, which results in the production of rdar (red, dry and rough) colonies of typical dark purple colour, with rough and dry surface and undulate margins (Milanov *et al*., 2015, Monteiro *et al*., 2011).The study was undertaken to investigate *in vitro* biofilm-forming capabilities of *P. aeruginosa* isolated from clinical specimens by two main different methods tissue culture plate (TCP) method and congo red agar (CRA) method and to compare these methods for biofilm detection. In addition to investigate the antimicrobial resistance profile among biofilm producing isolates.

2. MATERIALS AND METHODS

2.1. Specimen collection

The study was conducted at Rizgary Teaching Hospital and West Erbil emergency in Erbil City, Iraqi Kurdistan Region, during the first 6 months of 2017. A total of 300 specimens were collected from patients admitted to the hospitals. 96 clinical isolates of *P. aeruginosa* were isolated from various clinical samples including wounds and burns 40 (41.6%), ear discharge 17 (17.7%), blood cultures 15 (15.6%), urine 13 (13.5%), sputum 6 (6.25%), and abdominal fluid 5 (5.2%).

2.2. Bacterial identification

The Vitek-2 automated system by using Gram-Negative cards according to the manufacturer's instructions (GNI-20and 22) (bioMérieux, USA) (Vitek Systems Version: 05.04) was used for diagnosis to the species level. The isolated bacteria were stored in trypticas soy broth (TSB) with 40% glycerol at -70°C until used.

2.3. Detection of biofilm formation All bacterial isolates were tested by the following two methods for detection of biofilm formation (tissue culture plate method and congo red agar method):

2.4. Tissue culture plate method (TCP)

Tissue culture plate (TCP) assay described by Christensen *et al*. (1995) is considered the gold-standard method for biofilm detection. Isolates from fresh agar plates were inoculated in 10 mL of TSB with 1% glucose. Broths were incubated at 37°C for 24 h. The cultures were then diluted 1:100 with fresh medium. Individual wells of sterile 96 well flat bottom tissue culture plates were filled with 200 µL of the diluted cultures. Only sterile broths were served as blank to check sterility and non-specific binding of media. Similarly, control

organisms were also diluted and incubated. All three controls and blanks were put in the tissue culture plates. The culture plates were incubated at 37°C for 24 h. After incubation, to remove free-floating bacteria, gentle tapping of the plates were done. The wells were washed with 0.2 mL of phosphate buffer saline (pH 7.2) four times then were exposed to air-dry. The wells were then stained with 200 µL of 0.1% crystal violet for 30 minutes at room temperature. The plates were washed with distilled water to remove the unbounded dye, allowed to dry .The adhered stain was solubilized by addition 200µl of 95% ethanol. Optical densities (OD) of stained adherent biofilm were obtained by using micro ELISA auto reader at wavelength 630 nm. The experiment was performed in triplicate and repeated three times. Average of OD values of sterile medium were calculated and subtracted from all test values. ODs below 0.120 was considered as non-biofilm producers, 0.120 - 0.240 as moderate biofilm producers and more than 0.240 as strong biofilm producers(Bose *et al*., 2009, Hassan *et al*., 2011, Mathur *et al*., 2006).

2.5. Congo Red Agar method (CRA)

Freeman *et al*. (1989) had described a simple qualitative method to detect biofilm production by using CRA medium; which requires the use of a specially prepared solid medium -brain heart infusion broth (BHI) supplemented with 5% sucrose and Congo red. The medium was composed of BHI (Oxoid, UK) 37 g /L, sucrose (50 g /L), agar no.1 (Oxoid, UK) 10 g /L and congo red indicator (Oxoid, UK) 0.8 g /L.Congo red stain was prepared as concentrated aqueous solution and autoclaved at 121˚C for 15 minutes. Then it was added to autoclaved Brain heart infusion agar with sucrose at 55 ˚C. Plates were inoculated with test organism and incubated at 37˚C for 24 to 48 hours aerobically. Biofilm production was indicated by black colonies with a dry crystalline consistency. Brownish or reddish growth was considered as non biofilm producing organisms. The experiment was performed in triplicate and repeated three times (Hassan *et al*., 2011, Mathur *et al*., 2006, Bose *et al*., 2011).

2.6. Antimicrobial susceptibility

In vitro susceptibility testing of all collected isolates to a wide range of antimicrobials (ampicillin, cefepim, cefotaxime, cefoxitine, ceftazidime, cefuroxime, gentamycin, imipenem, meropenem, norfloxacin, ciprofloxacin, tobramycin, and colistin) was determined by Vitek-2 automated system which usually uses different Antimicrobial Susceptibility Test cards (AST-cards) according to the expected pathogens. The related cards were inoculated and incubated in the machine according to the manufacturer's instructions (Lima *et al*., 2017).

3. RESULTS

A total of 300 specimens were included in the study. *P. aeruginosa* was present in 96 of various clinical specimens. Biofilm production by CRA method and TCP methods was seen in 76(79.1%) and 84(87.5%) isolates respectively. 12(12.5) were negative by TCP, while 20(20.8) were negative by CRA method. Table1

Table1.The percentage of biofilm formation among *Pseudomonas aeruginosa* isolates (n=96)

Tissue culture plate method	
Biofilm producers No. (%)	Biofilm non producers No. (%)
84(87.5)	12(12.5)
Congo red agar method	
Biofilm producers No. (%)	Biofilm non producers No. (%)
76(79.1)	20(20.8)

As shown in Figure 1 among 96 *P. aeruginosa* isolates, in TCP method detected 78 as strong, 6 as moderate and 12 as weak or non- biofilm producers. Different results were observed by the CRA method, the number of strong biofilm producers were 65, moderate were 11 and weak or non-biofilm producers were 20.

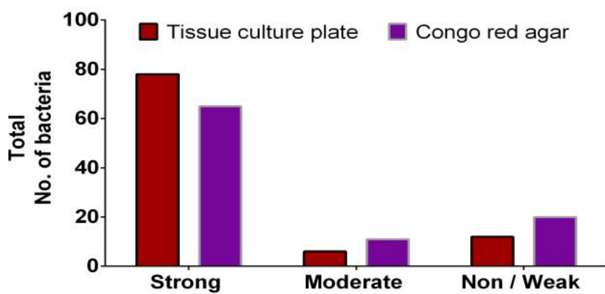

Figure1. Screening of *P. aeruginosa* isolates for detection of biofilm formation by tissue culture plate and congo red agar methods

Out of 13 antimicrobes tested, all ninety six isolates were found to be 100% sensitive to colistin. They revealed a high resistance rate for ampicillin, cefotaxime and cefuroxime (100%). Biofilm producer among *P. aeruginosa* displayed the highest resistance to cefoxitin 81(96%) followed by cefepim 65(77%), norfloxacin 62(73%), tobramycin 54(64%), gentamicin 53(63%), imipenem 51(60%), ciprofloxacin 48(57%), meropenem 44(52%), and ceftazidime 56(47%). The non biofilm producers after colistin showed lowest resistance to meropenem 6(50%) followed by imipenem, ciprofloxacin and gentamicin 7(58%), ceftazidime and tobramycin 8(66%), cefepim and norfloxacin 9(75%) and cefoxitin 11(91%). The antibiotic resistance pattern of *P. aeruginosa* was found higher in biofilm producers than in biofilm non-producers. Table 2

Table2. **Antimicrobial- resistance pattern of *P. aeruginosa* among biofilm producers and non producers**

Antimicrobial	Biofilm Producers No. (%)	Biofilm Non producers No. (%)	Total resistant No. (%)
Ampicillin	84(100%)	12(100%)	96(100%)
Cefotaxime	84(100%)	12(100%)	96(100%)
Cefuroxime	84(100%)	12(100%)	96(100%)
Cefoxitin	81(96%)	11(91%)	92(95%)
Cefepim	65(77%)	9(75%)	74(77%)
Ceftazidime	56(47%)	8(66%)	64(66%)
Ciprofloxacin	48(57%)	7(58%)	55(57 %)
Tobramycin	54(64%)	8(66%)	62(64%)
Gentamicin	53(63%)	7(58%)	60(62%)
Meropenem	44(52%)	6(50%)	50(52%)
Imipenem	51(60%)	7(58%)	58(60%)
Norfloxacin	62(73%)	9(75%)	71(74%)
Colistin	0	0	0

4. DISCUSSION

The major problem attributed with infections formed by biofilm producer bacteria is abundance of resistance to various antibiotics (Karthic and Gopinath, 2016). Production of an extracellular matrix is the hallmarks of a mature biofilm acts as a barrier for any antibiotics and increases resistance to these

antibiotics (Heydari and Eftekhar, 2015).In current study we tested 96 clinical isolates of *P. aeruginosa* for their ability to form biofilm by two phenotypic methods. TCP method could detect 84(87.5%) of 96 *P. aeruginosa* as biofilm producers compared to the CRA method 76(79.1%).Table1 Based on the current results, there was a high prevalence of biofilm production in our isolates. Also Bakir and Ali (2016) from Erbil (Iraq) reported that out of 34 Gram-negative bacteria including *P. aeruginosa* isolates were 25 (73.5%) isolates produce biofilm by TCP method but in CRA method 20 (58.8%) produce biofilm. Another finding obtained by Ahmed (2013) from Erbil who documented that out of (73) Gram-negative bacteria isolates in TCP method 28 (38.4%) isolates were produce biofilm as (strong and moderate) which were lower than present results. Furthermore, Lima *et al*. (2017) tested TCP to detect biofilm formation among *P. aeruginosa* isolates. According to their results, 75% of the isolates exhibited biofilm formation. Devaraj and Sajjan (2015) described maximum biofilm production in *P. aeruginosa* (100%) amongst Gram negative bacilli. TCP method was the most effective method and detected biofilm production in 93% of Gram negative bacilli. Study conducted by Rewatkar and Wadher (2013), employed both tube and congo red agar methods, he reported in his study that (54/60) *P. aeruginosa* isolates detected by CRA. In a study by Abdallah *et al*. (2011) from Cairo have reported that the percentage of biofilm production by *P. aeruginosa* was (50%) from urinary tract infection. Samant and Pai (2012) recommend the TCP method for biofilm detection in their study; they found a very high incidence of biofilm production in staphylococcal isolates (89%).From this study TCP method gave significant result 81.2% strong biofilm production as compared to the CRA method (67.7%). Figure 1 It was found that the TCP could differentiate between strong, moderate, and weak biofilm producers as compared to CRA. It is also reported as gold standard by other researchers (Lima *et al*., 2017, Samant and Pai, 2012, Karthic and Gopinath, 2016, Bakir and Ali, 2016).Hence TCP method was considered as standard method for additional interpretation of results. In consistent finding have been obtained by Bakir and Ali (2016) they presented that for biofilm production by TCP method as (moderate and strong) by *P. aeruginosa* was (81.8%) and CRA (72.7%).Study performed by Karthic and Gopinath (2016) noted that out of 20 clinical isolates of *P. aeruginosa* isolated from different clinical specimens, 35% and 25% as moderate and strong biofilm producers respectively, which was incomparable with our results. Indeed, *P. aeruginosa* possesses a variety of resistance mechanisms, which allow its survival under the action of antimicrobial or biocides, as well as make it an important nosocomial infection threat (de Almeida Silva *etal.*, 2017).It is evident from Table 2; there was a high frequency of resistance against all the frequently used antimicrobial agents. Biofilm producing *P. aeruginosa* isolates showed markedly high-level antimicrobial resistant to many groups of antibiotics as compared to biofilm non-producing isolates. This finding is important because therapy of patients with pseudomonal infections becomes more difficult when the isolate is biofilm producer, as biofilm is known to block the distribution of antibiotics. This observation is supported by various other researchers (Bakir and Ali, 2016, Karthic and Gopinath, 2016, Rewatkar and Wadher, 2013).The results in current study highlights the existence of association between antibiotic resistance and biofilm formation. Similar data have been recorded in the literature, Karthic and Gopinath (2016) showing high levels of biofilm in resistance isolates. This incidence is possibly attributed to gene transfer mechanisms within the biofilm environments, which is often acquired by transfer of genetic information from one organism to another as well as delayed diffusion of antibiotics inside the bacterial cell (Sahal and Bilkay, 2015). The highest resistance overall was observed against 3rd and 4th generation cephalosporins which is similar to Devaraj and Sajjan (2015) study which showed maximum resistance to penicillin (100%) and cephalexin (100%).This finding might be due to the inappropriate use

of antibiotics in this situation. According to our observation meropenem and ciprofloxacin were observed to be less frequently resistant, the present investigation is similar to the report of Karthic and Gopinath (2016). Both Bakir and Ali (2016) and Rewatkar and Wadher (2013) confirmed that all their isolates were sensitive to colistin which is in correlation with our results that showed none of the isolates were resistant to colistin ,meanwhile colistin is effective antibiotic against biofilm-forming bacteria.

5. CONCLUSION

This study documents a high incidence of biofilm productions were demonstrated among *P. aeruginosa* isolates.TCP method was considered as effective test for detection of biofilm formation and was also able to verify biofilm production by the *P. aeruginosa***.** Based on our findings we recommended TCP method as a screening method. Importantly, our *P. aeruginosa* isolates were observed to be resistant to most commercially used antimicrobials. This indicated a higher propensity among the clinical isolates of *P. aeruginosa* to form biofilm and there were a positive correlation exists between biofilm formation and antibiotic resistance.

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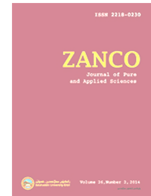

A New record of Sap feeding- beetles, *Nitidula flavomaculata* Rossi, 1790 (Nitidulidae: Coleoptera) from Iraq

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A B S T R A C T

. A New record of sap feeding beetles, *Nitidulida flavomaculata* Rossi,1790 as an important insect species in the field of entomology was described in Iraq. The species was collected from the animal corpses Carrion during the period of November 2015 until May 2016. The taxon is easily distinguishable, where the mandibles are bidenticles. The lacinia membranous with high density of yellow setose. 3th segment of labial palps elongated oval ,1.1 times as long as the 2nd . 4th segment of maxillary palps is elongated oval ,1.2 times as long as the 2nd segment. Antenna is capitate, 9th segment inverted flask shaped 1.2 as long as the 10th . Pronotum dark brown, yellow laterally. Tegmen extended and ellyptical, anterior part wide at posterior ,apical margin of median lobe broad, V-shaped . Aedegalapophysis long, tubular, 1.7 times as long as the tegmen. The important taxonomic parts have been photographed. Localities and date of the collection have been mentioned

1. INTRODUCTION

The Nitidulidae are commonly known as sap feeding-beetles a large family in the superfamily Cucujoidea in the suborder polyphaga that belongs to order Coleoptera. The family currently contains more than 4.000 species classified in ten subfamilies worldwide (Cline et al., 2014). There are eight species of Nitidulidae beetle recognized from the Western palaearctic region were introduced from outside of this area (Jelinek and Audision, 2007). A few nitidulids are predaceous and feed on scale insects or have symbiotic relationships with ants or other social Hymenoptera organism (Kirejtshuk, 1998). Hayashi (1978) indicated that the family have been found in various habitats feeding on flowers, fruits, and decomposed fungi and animal tissue tissues. Abogast and Throne (1997) studied many species of sap beetles that regards as agricultural pests weather in the field or inside stored products the dusky sap beetle on corn field Dobson and Erichson found some species on stored maize. Dowd (1991) mentioned the damage of sap beetles via either there feeding as a vector of fungi. In this family, three genera of *Nitidula* (Fabricius), *Omosita*(Erichson) and

Carpophilus(Stephens) include species that are useful in forensic entomology (Zanetti et al.,2013). *Nitidula* is important genus in the family which contain three to four generations years (Dowd and Nelson, 1994). *Nitidula flavomaculata* is on of important species in the family ;The species of has previously been reported from animal carcasses in Tehran and Lorestan provinces of Iran (Lason and Ghahari, 2013) . The main aim of this study was a detail description of the species

2. MATERIALS AND METHODS

The present paper is based on, 40 specimens which collected from the period of November 2015 till May 2016 from the animals corpse (goat, dogs and sheep) in many localities of Erbil governorate (Khostapa, Grdarasha and Darato). The specimens were placed in boiling water for 10-15 minutes to soften their parts. Then the parts were separated and put in 10% KOH, placed on fire with shaking for about (4-5) minutes for dissolving of lipids materials of the body and destroying the muscles. After that specimen. placed in distilled water for 2-3 minutes in order to A digital camera (Ucmas series microscope camera) was used to photographing the important parts. neutralize the alkali. The parts are placed in ethyl alcohol 25% then transferred to ethyl alcohol 50% , 75% and 100% respectively for two minutes for each concentration to dehydration of water , then placed in xylol for two minutes for translucency finally placed in Canada Balsam or DPX to support slides for subsequent examination under dissected microscope(Lane and Grosskey, 1993; Mawlood et al., 2016).The measured proportions of body parts are given in points of an eyepiece linear micrometer in a binocular microscope. And species were identified with the help of available literature of(Hinton, 1945; Hatch, 1961). The species confirmed by Dr. Michael Geiser the curators of Coleoptera (beetles)from University of Basel, Switzerland, London and Prof. Dr. Mohammed S. Abdul Rassoul in Iraq Natural History Research Center and Museum .

3. RESULTS AND DISCUSSION

Nitidula flavomaculata Rossi, 1790

Body: Oval,board ,subparallel, dark brown, feebly convex.Length 2.5-3.4 mm and width 1.1-1.5 mm. Dorsal surface with moderately dense, short , recumbent hairs

Head: Globular shaped, with narrow dark brown sutural edge, the punctures much coarser the facets of eyes. length 0.9-1.2 mm, width 1.0-1.3 mm.. Eyes dark yellow, oval.Vertex shiny black, weakly convex, with low fine of dense punctures. Coronal suture present. Frons shine black, slightly concave, with low density of fine punctures. Clypeus weakly concave, triangular, laterally with low dense of fine punctures and short yellow setae. Labrum (Fig.1a) transverse, pale yellow,0.3-0.5 mm length, posterior margin slightly emarginated at the middle, densely short pale yellow setose. Mandibles high sclerotized , a (Fig.1b) bidenticates , outer denticles long,3 times as long as the inner, , moler aria with density ,fine yellow setae. Maxilla (Fig.1c) pale yellow, lacina elongated oval apical and outer margin densely high short yellow setose, 1-3 maxillary palp cup shaped, 2nd segment 1.2 times as long as the ^{3rd} segment, 4th segment elongated oval ,1.2 times as long as the 2nd segment. Labium (Fig.1d) brown, 2nd segment of labial palp cup shaped 4 times as long as the 1st segment , 3rd segment elongated oval, 1.2 times as long as the 2nd segment, each segments with 2-4 short setae. Antenna (Fig.1e) brown, capitate, 11 segmented , 1.0 – 1.4 mm long, 1st segment elongated oval, 1.3 times as long as the 2nd segment, 3rd segment cylindrical , 1.2 times as long as the 4th

segment, 9th and 10th segments cup shaped,10th segment 1.2 times as long as the 10th , 11th segment nearly triangular , 1.1 times as long as the 9th segment.

Thorax :Pronotum in dorsal view shine dark brown, laterally yellow, surface randomly irregular fine punctures and moderate short brown setae, the anterior margin moderately concave, posterior margin nearly straight; the anterior and posterior with row of pale yellow setae, anterior and posterior angle slightly acute,pronotum with punctures as fine or finer than those of elytra , disk of pronotum more or less flat.Procoxal cavity open, prosternal process nearly globular. Scutellum shine yellow, semi rounded, surface sparsely fine punctures. Elytra (Fig.1f): shine brown, flat, nearly 3/4 of basal part dark brown, surface densely short brown setose with fine punctures .Epiplural straight, dark brown, with fine punctures. Hind wing pale white, veins weakly developed , stigma oval, yellow. Fore legs(Fig. 1g) brown, for coxa cone shaped, fore legs isosceles triangle ,apical with 2 short spurse ,apical part with a row of spines and without spurs, for tarsus 5 segmented, 1-2 segments cup, 3rd segment bilobed ,4th segment is the smallest hidden in bilobed of 3rd segment , 5 segment tubular , 4 times as long as the 1st ,the segments 1-3 with high densely ,short, fine yellow setose. Claw simple , moderately curved. Middle legs resemble to fore legs except ;the coxae nearly spherical. Hind legs resemble to fore legs except ;the coxae boat shaped , hind tibia narrow and longer **Abdomen** : Dark brown, six segmented ,covered with high densely brown setose , 1-4 abdominal sternites rectangular , 1st 1.2 as long as the 1st , the segments 2, 3 and 4 nearly same length , 5th segment trapezoidal shaped , 6th segment cup shaped .1st -5th tergites nearly triangular, 1st -5th abdominal tergites rectangular, 6thtergite nearly triangle shaped. 9th abdominal sternite dark yellow, U- shaped ,1/2 posterior part moderately fine brown setose, posterior margin sparsely yellow setose, lateral arms tubular ,apex rectangular. 9th abdominal tergite(Fig.1g) pale brown, posterior part bilobed nearly triangle, sparsely brown setose, anterior part long tubular shaped

Male genitalia :Aedeagus (Fig.1i, j) pale brown, length 1.3-1.7 mm long, From dorsal view (Fig. 1i), the tegmen 0.4-0.6 mm length ,extended and ellyptical, anterior part wide at posterior, with thin connection on medio-proximal, other lateral margins sclerotized from base to distal, median lobe contoured with highly sclerotized and U-shaped thin band, lateral margins distinctly arched through apical. Aedegalapophysis tubular shaped , 0.9 - 1.2 mm long , 2 times as long as the tegmen . From lateral view (Fig.1 j), Tegmen sinuous, almost parallel through proximal, aedegalapophysis slightly curved and wavy on anterior half; median lobe arched and almost parallel with tegmen. Species of sap beetle, *N. flavomaculata* Rossi**,**, is commonly distributed throughout the Turanic-Mediterranean basin extending easterly to Turkey and southern parts of Iran (Mifsud and Audisio, 2008). The species on a human corpse has rarely been reported. This beetle is considered as an insect of forensic significance in the world and it has previously been reported from human corpses in line with the present study (Sims and Fothergill ,2014). This description agreement with study of (Hinton, 1945) the punctures much coarser the facets of eyes. Disk of pronotum and elytra more or less flat. (Hackson, 2017) mentioned that the pronotum very dull , flat. Elytra usually with large irregular reddish-yellow patch at the base and second ,elongated patch over the suture beyond half-way which may be united with the basal patches. Ozdemir and Serto (2008) mentioned that the tegmen in dorsal view extended and elliptical, median lobe contoured with highly sclerotized and U-shaped thin

band, lateral margins distinctly arched through apical, apical margin broad V-shaped, anterior angles sharp, anterior part with a couple of horn-like projection.

Examined specimens: The specimens were collected from animal corpses (goat, dogs and sheep) in different localities of Erbil governorate- Iraq : Grdarasha,30.11.2015; 22.12.2015;Qushtapa, 10 3.2016; and Darato,5.4.2016.

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Conflict of interest

There is no conflict of interest

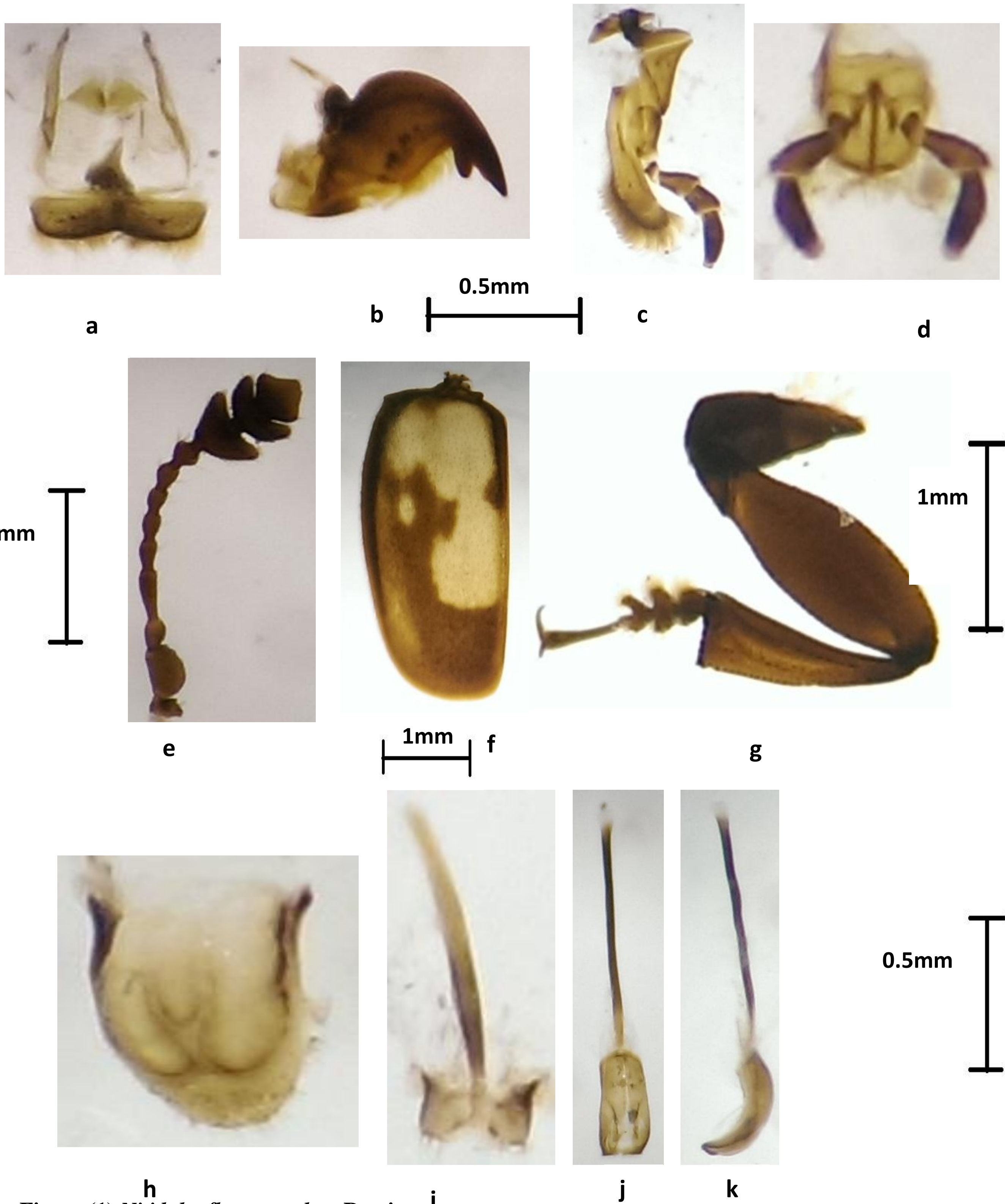

Figure (1) ***Nitidula flavomaculata*** **Rossi**

a. Labrum b. Mandible c. Maxilla d. Labial palps e.Antenna f. Elytra g. Fore leg h.9th abdominal sternite i. 9th abdominal tergite j. Aedeagus (dorsal view) k. Aedeagus(lateral view)

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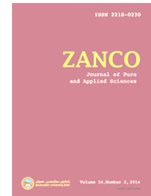

Some heavy metals assessment in frozen chicken meat sold in Erbil local markets

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ABSTRACT

The present work was conducted to evaluate the quality of frozen chicken imported to Iraq regarding the heavy metal content of six elements Iron (Fe), Manganese (Mn), Magnesium (Mg), Calcium (Ca), Zinc (Zn) and Lead (Pb) in ppm were determined in three different body parts i.e. breast, wing and thighs of Brazilian and Turkish origins. These heavy metals were determined using X-ray fluorescence spectrometer (XRF). The contents were compared with the internationally allowed levels for safety reasons. However, Iron, Manganese, Magnesium, Calcium have been within the range, the Zn and Pb have exceeded the internationally allowed limits by WHO in Brazilian chicken. Both wing and thigh muscles, especially in Brazilian revealed higher proportion of Zn than permissible limits (10-50 ppm) which was allowed by ANZFA in comparison with other breast tissues. Chicken of Turkey is reported to be safer than that of Brazil. People are better to utilize domestic chicken rather than the imported one as it could be contaminated with heavy metals that come from commercially-produced foods.

1. INTRODUCTION

Chickens represent significant diet abundant with numerous vital nutrients like proteins, oils and vitamin D (Schonfeldt& Gibson, 2008).Heavy metals vary in their levels in different birds' species according to several parameters. These metals were spotted in various body parts including, blood, skin, lungs and feathers in various proportions (Abduljaleel*et al.,* 2012). Provincial and domestic amounts of heavy metals affect the functional and physical integration of ecosystems (Abduljaleel*et al,* 2012).

Poultry within agricultural enterprises of farmers which involve flocks always criticized for not receiving adequate health measures as a result of the vulnerability of their birds to ecological man-made actions and air pollution (Schwartz, 1994). The latter leads to consequent precipitation of these pollutants inside animal stocks which are consumed directly by human beings or and indirectly within the food chain. Accordingly, the risk associated with exposure to heavy metals present in food product had led to a wide spread concern in human health.

The concentration of heavy metals in the internal tissues of chicken has been extensively studied (Hussain *et al.,* 2012; Hamasalim and Mohammad, 2013; Hassanin*et al.,* 2014). Bioaccumulation of heavy metals in the tissues

of poultry has generated public health concerns due to the lethal and sub-lethal effects of their accumulation in the food chain (Burger *et al.,* 1994). Some heavy metals alert potentially toxic effects i.e. As, Cd and Pbin addition to trace elements like Fe, Mg, Cu Zn ,Se, Ni and Co (Duruibe*et al.,* 2007). Data on trace element levels in chicken and other domestic birds in Iraq are still scarce. The accumulation of heavy metals in avian bodies has recently drawn some attention upon lethal and sub-lethal effect of their accumulation a part from the convenience use of the food chain bioaccumulation studies (Burger *et al.,* 1994). Due to the fact that these contaminant sare abundant in the ecosystems, accumulation to poisonous levels is not totally avoidable. Nevertheless, minimizing them is crucial to lessen both the direct impacts on bird well-being and indirect impacts on man well-being (Scan, 2003). The current study aims to determine the levels and distributions of certain heavy metals which are Fe^{+3}, Mn^{+}, Mg^{+2},Zn^{+2}, Ca^{+2} and Pb^{+2} in breast, wing and thigh of frozen Brazilian and Turkish chicken consumed in Erbil city.

2. MATERIALS AND METHODS

2.1. Sample Collection

Ninety samples of Brazilian and Turkish frozen chicken tissues have been randomly taken from domestic stores in Erbil (45 samples for each) between May to August 2015. The samples involved 15 wings, 15 breasts and 15 thighs . Because people in Erbil and Kurdistan prefer these types of chicken meat specially they are good source of protein and have suitable price, so the meat cuts collect from local markets of different places in the province and stored at freezing degree until tested in the chemical lab. of the agriculture college.

2.2. Sample preparation

A 10 gm±0.2 sample of every part is weighed up via a sensitive balance, put in oven at 70º C for 24 hrs. Then, it is grounded to powder by a mortar. For each part, two grams were utilized to analyze their metals residues content by X-ray fluorescence spectrometer device.

2.3. Metal Analyses

X-ray fluorescence spectrometer(XRF), Skyray 9000, portable device (USA.).(Rajib et.al.,2016 and Hutton et.al.,2014). The XRF spectrometry is an instrumental analytical approach that is capable of determining the makeup of solid and fluid substances from minimal, and the device present at chemistry lab. in the agriculture college . This approach is also utilized for the direct examination of both solid and liquid substances. The samples have been exposed to the X-ray to stimulate the atoms to emit a distinctive radiation for specific metals. The wave lengths of such a distinctive radiation differs from one element to another. This phenomenon forms a basis for the qualitative examination of elements. The power of the distinctive radiation of metals is measured to its concentration that allows for the qualitative examination.

2.4. Statistical Analyses

Results were subjected to statistical analysis using Statistical Analysis System package (SAS) Version 2002-2003 software and statistical significance was set at $p \leq 0.01$and $p \leq 0.05$.

3. RESULTS

the results are shown in table 1 indicating the significant difference in level of all elements except magnesium, the mean level of six various elements in the Brazilian chicken was remarkably greater ($p \leq 0.05$) than the Turkish one (Table 1). The Fe+ levels were equal in the three cuts of both type of chicken. Mn+, however, was remarkably greater ($p \leq 0.05$-≤ 0.01) in the wings of Turkish chicken (43.12±4.53) than in Brazilian ones (28.17±2.27) and it was remarkably greater than the global acceptable level (Fig. 2). Mg

and Ca ions means were equal in both types of chicken regardless to the cuts of the body. While Zn+2 proportions appeared similar in both types of chickens except in thigh cuts appeared three folds over and significantly higher ($p \leq 0.01$) in Brazilian than in Turkish one. Unexpectedly, Pb concentrations were remarkably greater in the Turkish chicken ($p < 0.05$) than the Brazilian one, especially in the wing, however it was beneath the global acceptable levels (Table 1).

Table 1. Illustrates the means and standard deviations of Fe+3, Mn+2,Mg+2, Ca+2, Zn+2 and Pb+2 as ppm spotted in various parts.

metals	origin	Breast muscle	Wing muscle	Thigh muscle
Fe %	Brazilian	0.09±0.01	0.09±0.01	0.09±0.01
	Turkey	0.09±0.01	0.09±0.01	0.09±0.01
IPL	-	-	-	-
Mn (ppm)	Brazilian	37.08±2.52	28.17±2.27*	39.68±2.39
	Turkey	38.96±3.24	43.12±4.53	44.60±3.03
IPL		6.5	6.5	6.5
Mg (ppm)	Brazilian	0.02±0.00	0.06±0.03	0.02±0.00
	Turkey	0.02±0.00	0.03±0.00	0.02±0.00
IPL	-	-	-	-
Ca (ppm)	Brazilian	1.66±0.03	1.78±0.05*	1.58±0.04
	Turkey	1.64±0.04	1.64±0.04	1.54±0.05
IPL	-		-	
Zn (ppm)	Brazilian	32.19±4.65	88.27±9.91	131.27±24.01*
	Turkey	30.30±5.79	86.13±10.71	47.73±4.61
IPL	10-50	10-50	10-50	10-50
Pb (ppm)	Brazilian	0.44±0.03	0.84±0.06*	0.43±0.02
	Turkey	0.39±0.01	0.63±0.02	0.42±0.02
IPL	1	1	1	1

International Permissible Limits (IPL according to ANZFA,2001); (*): Significant ($p \leq 0.05$); (**)$p \leq 0.01$).

4. Discussion

Heavy metals bioaccumulation in avian meat and liver was examined by a number of studies (Kirkpatrick and coffin, 2006; Santhi*et al.,* 2008; Blanco-Penedojl *et. al.,* 2009). Heavy metals bioaccumulation in birds is an unavoidable universal event especially in developed nations. Metal remains are noticed in some segments of bird's body such as plumages (Abduljaleel et al.,2012). These metals are regarded present pollutant that may not easily be demolished by thermal action , to which degree their ability is promoted to reach and effect , in the food net work ,human(levensen and Barnard , 1988). Iron levels in all segments of both origins show similarity in being below the acceptable levels in rodex system form of hemeproteins and non heme-enzymes because it is a crucial component in all living creators including invertebrates and humans (Kanakaraju*etal.,* 2008).

Hence, there are the low levels of Fe ion below the universal levels in such imported varieties if compared to that of the red meat(Pennington *et al.,* 1998). Universal standards have considered all segments of the chicken as a whole rather than a particular segment. variation in the levels of such metals in these

three segments were obvious. Each may indicate various deposition density or it might be attributed to the lesser muscle mass in wings when contrasted to breast and thighs. This might serve as an indicator for the fact that consuming breast and thighs is safer than consuming wings. However, Mn levels were remarkably greater than acceptable levels in all chicken muscles(6.5) ppm in accordance with Becking and Chen (1998). Although, the average Mn levels in Turkish variety wings displayedan exception since it was remarkably greater if compared to the Brazilian one. This may mean the presence of a greater Mn level in feed used up by Turkish variety. Though, this result may require more enquiry to discover the exact cause. Although the consumer dietary exposure of Mn is 67 µg/kg body weight for adults, 101 µg/Kg body weight for young people (FSIS, 2004) the daily consumption of little or trace Mn levels is vital for humans to grow and stay healthy. Mn deficiency may trigger nervous ailments (Dermirezen and Uruc, 2006).There are no acceptable levels of Ca in beef mutton and chicken which are vital intracellular cation that works as a second messenger in more than one signal transduction cascades (Irfana*et al.,* 2004). Usually, both Mg^{+} and Ca^{+2} element in both chicken meat are regarded electrolytes that are valuable for the body (WHO/FAW) indicated decreased levels (0.02 and 1.6ppm, respectively). Mg is regarded among the seven indispensable macro-nutrients vital as much as or greater than 100mg/day. Humans accommodate about 20-28 mg of Magnesium in which more than half the quantity is deposited in the system while the remaining is goes to muscles, soft tissues and physical fluids (Krause's food and nutrition therapy,2008). In spite of the known significance, no explanation is determined in the current research. Trace Zn may trigger troubles because excess amounts of Zn may negatively affect human health since it is an intestinal irritant found in the atmosphere (ATSDR, 2004). The remarkable greatest ($p \leq 0.05$) Zn levels was found in the muscles of both varieties predominantly in thighs and over the acceptable levels (ANZFA, 2001). Still, in the Brazilian variety it was remarkably greater than the other one in wings and thighs. These differences may not offer clear explanations ;however ,genus or/and species differences cannot be ignored. These outcomes; are consistent with the latest study of Hamasalim and Mohammad(2013), Demirbas (1999) ,Sari *et al.,* (2008), Abduljaleel*et al.* (2012). Lead is responsible for reducing cognitive progress and intellectual competence in kids and it elevate blood pressure and contributes tocardiovascular disease in adults (Commission of the European, 2002). Livestock takes the lead mainly from the air, water, nutrients, cooking tools and food wrapping. Lead levels are below the acceptable levels in both chicken meat (0.5-1 ppm) (Young, 2005) which means that they are safe to be consumed.

4. Conclusion

The result of the present findings showed. Turkish chicken meat contained lower levels of heavy metals compared to Brazilian chicken meat. These levels of elements in Brazilian chicken meat could result from contamination of the feed , water source , the environment ,the processing and the packaging. Therefore people that consume Brazilian chicken meat in preference to Turkish chicken meat are likely to be exposed to higher metal levels. Therefore anthropogenic factors that proliferate heavy metals in the environment should be discontinued and discouraged and further studies must be done to determine these metals concentration in the internal organs of these types of imported chicken.

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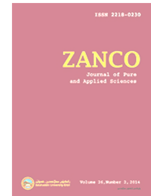

Phytotherapeutics: As anticipating substitutes to synthetic drugs in combating antinematicidal-resistant gastrointestinal nematodes of small ruminants

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A B S T R A C T

The plant kingdom embraces a plethora of various phytomedicines which have been exploited for thousands of years by conventional pastoralists, herbalists and healers as a part of ethno-veterinary medicine against livestock nematodiasis. The role of medicinal plants has been diminished after the marketing of synthetic thiabendazole (first generation of benzimidazole group) in 1961. Virtually, the magnitude of herbal medicines has been substantiated again in recent decades due to the rapid emergence of antinematicidal resistance among the parasitic nematode populations as a result of discriminate use of the synthetic chemotherapeutics worldwide. Furthermore, other promoted alternatives viz. biological control, immunization, grazing management, nutritional supplements and genetic approaches have not been commercialized yet due to some practical bottlenecks. In this article, the significance of medicinal botanicals and the performed studies in the field of phytotherapy toward antinematicidal-resistant alimentary tract nematodes parasitized ovine and caprine have been reviewed elaborately.

1. INTRODUCTION

It has been narrated and postulated that various plants were utilized as therapeutic agents for the first time by the distinct primate ‘Neanderthal’ (*Homo neanderthalensis*) between 60,000-80,000 years BC in the Kurdistan Region of Iraq (Agelarakis, 1993; Sommer, 1999; Hamad, 2012). Records attribute the use of medicinal plants to prehistoric Mesopotamian Civilizations (land between the two rivers of Tigris and Euphrates), in particular the Sumerians who dwelt modern over 5,000 years ago (Levetin and McMahon, 2003). It is noteworthy to mention that exploitation of herbal remedies was practiced by Chinese herbalists (Ergil *et al.,* 2002) and Indian botanists (Aggarwal *et al.,* 2007) around 2700 BC and 1900 BC, respectively. Ancient Greeks and Romans as well as Muslim scientists have also contributed to the use of phytomedicines (Iqbal *et al.,* 2001). The recent decades have witnessed a distinct development in herbology by employing modern pharmacological techniques

in the extraction of crude materials and active constituents of several plants (Niezen *et al.,* 1998). For this purpose, the adulticidal, larvicidal and ovicidal efficacy of different herbal medicines have been validated and documented by investigators in Asia, Africa and Latin America to fight gastrointestinal nematodes of small ruminants (Iqbal *et al.,* 2001; Githiori, 2004; Carvalho *et al.,* 2011; Hamad, 2012). Evidently, justifications behind the use of botanical medicines are attributed to rapid development of antinematicidal resistance among alimentary tract nematodes against renowned synthetic dewormers around the world (Kaplan, 2004; Jabbar *et al.,* 2006). Moreover, the presence of some drawbacks and side effects including environmental pollution, toxicity and residual impacts have reckoned other reasons to employ botanical dewormers in place of allopathic drugs (Waller *et al.,* 2001). Researchers in the field of phytotherapy have accomplished significant levels of success. However, more sophisticated phytopharmacological, phytotoxicological and biochemical assays are needed for the commercialization of herbal remedies (Hammond *et al.,* 1997).

2. Antinematicidal magnitude of medicinal plants

Phytotherapy is a traditional medicinal practice relying on the utilization of ethnobotanicals and their extracts to treat various human and animal diseases. Conventionally, thousands of indigenous plants are being used by herbalists and livestock raisers in developing countries to cure infections with gastrointestinal (GI) nematodes in small ruminants (Akhtar *et al.,* 2000; Waller *et al.,* 2001). On the other hand, scientific validations and documentations of indigenous medicinal plants against GI nematodes have witnessed a considerable progress, particularly in the southern hemisphere (Waller, 2006). When compared to orthodox medicinal practices, botanical medicines possess some unique properties viz. low toxicity, less residual effects, very slow evolution of resistance as well as being environmentally friendly (Waller *et al.,* 2001). Moreover, the plant kingdom is rich with phytomedicines in addition to their relatively low costs, accessibility and acceptability by farmers mainly in developing countries. Routinely, different parts of the plants are being used including the whole plant, leaves, barks, fruit, root, rhizomes, vinegar, aerial parts, cake, bulb, pulp, latex, wood, stigmas, stem, oil, wine, gum, shoots, buds, seeds, flowers, inflorescence…etc (Iwu, 1993; Van Wyk *et al.,* 1997; Akhtar *et al.,* 2000; Hamad *et al.,* 2012; Chagas, 2015). It is noteworthy to mention that medicinal botanicals contain various active phytoconstituents such as phenols, flavonoids, tannins, alkaloids (approximately present in 158 botanical families), terpenoids, glycosides, exudates…etc. (Cowan, 1999; Chagas, 2015). Regarding the current problem which is associated with the development of antinematicidal resistance (AR) among GI nematodes in a wide range of countries, herbal medicines could be the apposite substitute to overcoming this dilemma. Expectantly, these phytochemicals will occupy their places in the inventory of effective antinematicidals in the near future (Jabbar *et al.,* 2006).

3. Development of resistance against synthetic drugs

Resistance among parasitic nematodes has emerged against the renowned broad-spectrum anthelmintics which include benzimidazoles/pro-benzimidazole, tetrahydropyrimidines and macrocyclic lactones (avermectin/milbemycins) in almost every country especially in areas where small

ruminants are intensively being raised (Kaplan, 2004; Beech and Silvestre, 2010). There are some reports about field detection of AR against monepantel (a recent dewormer produced by Novartis Animal Health INC. under the commercial name Zolfix which marketed in 2009) in New Zealand (Scott *et al.,* 2013) and Uruguay (América *et al.,* 2014). In this regard, investigators in the field of parasitology have laid blame on some factors that contribute to the escalation of AR. These enhancing factors may include the indiscriminate use of drugs, recurrent deworming, sub-standard dosing (prophylactic doses), mono-drug strategy and poor quality drugs in developing countries (Dorny *et al.,* 1994; Hoekstra *et al.,* 1997). Dissemination of AR against commonly used dewormers has been presented in table 1.

4. Rampancy of resistance (selected countries)

AR has been evidently rooted in almost every country in the world. Undoubtedly, this problem is most serious in areas where small ruminants are being reared intensively such as Australia, Uruguay, South Africa, New Zealand and Pakistan. The dissemination of AR in some countries is shown in table 2.

5. Phytopharmacology and Phytotoxicology of medicinal plants

Several phytopharmacological and phytotoxicological considerations should be elaborated regarding the utilization of ethnobotanical extracts for treatment of parasitic and non-parasitic ailments. While ethical medicinal products (pharmaceuticals) have been widely investigated pharmacologically and toxicologically by commercial companies, the studies on medicinal plants are still rare (Couatre, 2004), particularly in the field of veterinary medicine. Nevertheless, documentation and validation of home-grown phytomedicines (as a substitute to orthodox drugs) against GI nematodes of small ruminants are being executed extensively by South Latin American, Asian and African researchers, yet their trials mostly encompass clinical studies without comprehensive phytochemical investigations (Chagas *et al.,* 2014).

In order to be more reliable and safer for clinical pharmacology use, phytotherapic substances should undergo pharmacokinetic and pharmacodynamic studies. In this regard, in vitro and in vivo assays to assess antinematicidal (adulticidal) activity of trialed plant extracts should be performed properly (Hamad, 2012). Moreover, herbal extracts or their active ingredients should be evaluated for their potency to prevent egg hatching and larval development (in vitro techniques) of nematodes (Max, 2010; Tayo *et al.,* 2014). It's worth mentioning that medicinal plants can impair egg hatching and not egg embryonation (Hamad, 2012). Pragmatically, likely positive impacts of plant extracts take place such as reduction of parasite burdens. This, in turn, leads to less egg contamination of pastures, minimizing the likelihood of re-infection with infective stages of nematodes (Max, 2010; Tayo *et al.,* 2014). Needless to say, the adulticidal, larvicidal and ovicidal potency of a plant extract in vitro are not a reflection of its efficacy in vivo owing to some pharmacological considerations such as, ruminal pH, destruction of active constituents and biodegradation by rumen flora, bioavailability, absorption, metabolism and excretion (Pervez *et al.,* 1994; Peneluc *et al.,* 2009; Katiki *et al.,* 2012).

In spite of the importance of botanical medicines to control antinematicidal-resistant nematodes, the toxicological aspects and safety concerns need not be ignored when any medicinal plant extract is prepared for animal treatment. In this regard, we should remember the words of Paracelsus, the father of modern toxicology, who said "All substances are toxic with the dose making the difference" (Borzelleca, 2000). Certainly, this statement

goes through with the herbal medicine extracts because most of the in vivo herbological experiments have revealed that the majority of phytomedicines are dose-dependent in their toxic effects and antinematicidal efficacy especially when they are used as crude extracts (Hamad, 2012).

While no mortality cases were recorded, sheep exposed to 4 g kg 1- (high dose) crude aqueous-methanol extract (CAME) of Nicotiana (N.) tabacum had showed clinical signs of central nervous system (CNS) intoxication including, restlessness, staggering gait, slight salivation, recurrent recumbency and slight nasal discharges, while those exposed to a low dose (2g kg-1) of the same plant has not conduced to develop any symptom of CNS intoxication. Statistically speaking, no significant difference (P>0.05) was observed between both therapeutical doses (Hamad, 2012; Hamad *et al.,* 2012; Hamad *et al.,* 2014).

In this regard, further initial trials have been advocated such as, determining acute, sub-chronic and chronic toxicity in experimental laboratory animals (rats and mice). Moreover, teratogenic, carcinogenic, neurotoxic and mutagenic influences have been suggested to be investigated in targeted animals (Camurça-Vasconcelos *et al.,* 2005).

6. Are phytomedicines inevitable alternatives?

The adoption and popularity of medicinal plants are increasing promptly among developing nations and even in highly industrialized countries particularly after the utilization of state-of-the-art techniques to conduct contemporary scientific investigations in the field of herbology. This technological revolution in the last few decades has enhanced sufficiently in recognizing a plethora of different phytochemicals and analyzing their active constituents to familiarize and convince people to use them in the domain of human and veterinary medicine (McGaw and Eloff, 2008). Habitually, poor pastoralists in most remote rural areas of developing countries have been adapted to deworm their livestock with herbal remedies as a result of either high expenses of allopathic antinematicidals (western pharmaceuticals) or unavailability of these vermifuges in their territories (Mathias, 2004). Fortunately, these ethnoparasitic practices have conduced to a much reduced emergence of antinematicidal-resistant individuals among GI nematode populations which reflects an increase in the number of parasites in refugia (those that are not exposed to synthetic drugs such as larvae in pasture).

It is noteworthy to mention that the main reason for the slow development of AR in those remote rural areas may be due to the presence of a number of active constituents in one plant which, in turn, can act in various mechanisms (Athanasiadou *et al.,* 2007). Most probably, the AR will develop rapidly as a consequence of the wide use of an active ingredient isolated from a given medicinal plant, particularly, if its antinematicidal potency has a single pharmacodynamic (Chagas, 2015). Moreover, the efficacy of some medicinal herbs as effective agents to control antinematicidal-resistant nematodes could be attributed to the occurrence of a sort of additive or synergistic action between active chemical compounds (working at single or several targets) within a particular plant and not between different medicinal plants. On the other hand, as pointed out by Tyler (1999), this synergistic or additive pharmacological impact can be useful by eliminating the problem of unfavorable side effects associated with the predominance of a sole xenobiotic chemical compound in the body. In this respect, the study of Hamad (2012) did not reveal any difference (P > 0.05) in the synergistic or additive effects of antinematicidal potency of some combined medicinal plants (details in the next subtitle).

In order to maintain drug susceptibility and minimize the percentage of resistance among antinematicidal-resistant GI nematodes, it will be valuable to add or replace 5-10% of a flock (harboring resistant nematode worms) with small ruminants from the rural areas where ethno-veterinary botanical medicine is

being practiced where access to synthetic antinematicidals is impossible (Sindhu *et al.,* 2010). This process can dilute resistant roundworm populations and reduce the speed of AR evolution. However, this hypothesis is almost unfeasible in developing countries due to several bottlenecks and constraints associated with culture, education, costs and the likelihood of transmission of other non-parasitic infectious diseases.

On the other hand, the idea of "preserving in refugia nematodes" to sustain susceptible nematodes on a farm may be done by another way that has been suggested by some researchers. Basically, the policy depends on leaving 10% of the stock untreated leading to the production of susceptible eggs by unexposed worms to synthetic antinematicidals. This strategy, while difficult to be conducted, but using of FAMACHA Anaemia Guide Chart (developed by Faffa Malan in South Africa) can facilitate the selection of animals carrying the most parasitic burden for deworming purposes. In light of this chart, small ruminants in stage 3, 4 and 5 of anaemia should be treated and those in stage 1 and 2 will be left untreated because the parasitic load is low (Macedo *et al.,* 2010). Unfortunately, some of the drawbacks of FAMACHA Chart include its applicability for haematophagous nematodes such as Haemonchus contortus (also for trematodes like liver fluke) and the fact that it may not be available everywhere especially in developing countries.

Nevertheless, for non-blood sucking GI nematodes, the five- point check (an extension of FAMACHA system), which has been developed in South Africa as well, could be applied for determining the deworming strategy to retard AR. Another policy that has been suggested by some researchers is associated with the use of combined synthetic drugs and phytomedicines (Dupuy *et al.,* 2003). This recipe, however, has not been tried yet (Cala *et al.,* 2014).

AR become a phenomenon on all the continents especially in areas where tamed small ruminants are being raised intensively, and therefore, any attempt to avoid development and prevalence of resistance among GI nematodes is too late (Waller, 1997). Hence, the utilization of ethnobotanicals to overcome the problem of resistance dissemination among antinematicidal-resistant GI nematodes might be an anticipated substitute in the near future.

Comprehensive studies on the effectiveness of medicinal plants to control the prevalence of antinematicidal-resistant GI nematodes of livestock are very rare (Hamad, 2012). The reasons behind the ignorance of employing ethnobotanicals may be owing to the focus on other strategies (non-chemical methods) such as, biological control, grazing management, genetic approaches, nutritional supplementation, and immunization particularly in the northern hemisphere. Unfortunately, these strategies have achieved a limited degree of success and they are still far from the ambitions of livestock raisers. On the other hand, while the popularity of medicinal plants is increasing in human medicine, especially in Germany (about 67000 medicinal plant extracts and products are being used) (Foster, 2009.

It is noteworthy to mention that antinematicidal-resistant pathogenic nematodes are more prolific, more pathogenic, have enhanced settlement rates in the host, and have raised longevity of the free-living stages in pasture (Kelly *et al.,* 1977). It has also been reported that eggs of resistant strains will embryonate and hatch in higher concentrations of antinematicidals than those of susceptible parasites (Le Jambre, 1976).

Thus, these defence properties of resistant pathogenic helminths have emboldened investigators in the field of nematology to adopt the aforesaid alternative strategies. Until now however, unfavorable results have been obtained in field. Additionally, some of these policies including vaccination, biological control using nematophagous fungi, and genetic approaches remain under investigation (Stear *et al.,* 2007).

In contrast, under the umbrella of non-chemotherapeutic approaches, phytotherapy is

presently a motivating area of research anticipated to be a promising reliable alternative to restrict rampancy of ecto-endo parasites in the near future. Researchers, especially in the Indo-Pakistan subcontinent and in other Asian, African and South Latin American countries, have conducted several studies on the potency and validation of indigenous medicinal plants to mitigate the parasitic burden (Akhtar *et al.,* 2000; Waller *et al.,* 2001; Carvalho *et al.,* 2011). Obviously, their trials were done randomly without differentiation between antinematicidal-resistant and susceptible parasites.

The study executed by Hamad (2012) is perhaps the first attempt to utilize extracts of some native medicinal plants in Pakistan against antinematicidal-resistant Haemonchus contortus in sheep. His study has revealed the efficacy of CAMEs of N. tabacum leaves, Azadirachta (A.) indica seed kernels and combined N. tabacum leaves and A. indica seed kernels through performing the fecal egg count reduction test (FECRT), egg hatch assay (EHA), and adult motility test (AMT).

Concerning the mean fecal egg count reduction percentage [FECR (%)], the results exhibited 88.6, 85.14, 94.59 for N. tabacum leaves, A. indica seed kernels and combined N. tabacum leaves and A. indica seed kernels, respectively. In this regard, we have to point out the recommendations of W.A.A.V.P (second edition) edited by Wood et al. (1995) that suggest that any dewormer with FECR% (98) is deemed highly effective; FECR% (80) and above is effective; whilst FECR% less than (80) is not commended for use. So based on the study carried out by Hamad (2012), Hamad et al. (2012) and W.A.A.V.P recommendations, the aforementioned phytomedicines are effective against antinematicidal-resistant nematodes.

Moreover, the in vitro assay (EHA) was also an indicator for potency of the aforesaid medicinal botanicals for their ovicidal efficacy through the calculation of LC50 values and 95% fiducial confidence interval (Lower-Upper). The ranks of the tested plants in this study were determined as shown in table 3. Regarding the other in vitro assay (AMT), the adulticidal activity of the abovementioned herbs was determined depending on the comparison between the three factors of time, concentration, mortality where LC50 values were calculated as well. The analysis is demonstrated in table 3.

It should be mentioned that the effectiveness of CAMEs of N. tabacum leaves, A. indica seed kernels and combined N. tabacum leaves and A. indica seed kernels on antinematicidal-resistant H. contortus through utilizing FECRT, EHA and AMT was significant. These botanicals contain detrimental chemicals against adult worms and their ova. Furthermore, the study also revealed that the trialed medicinal plants had prevented the hatching of eggs, but not their embryonation. As a result, further studies are required to elucidate this mechanism particularly the comparison between eggs recovered from susceptible and resistant nematodes to renowned antinematicidals.

The presence of a synergistic combination between natural phytotherapeutics to control antinematicidal-resistant GI nematodes has not been proven. In fact, studies on such topic are seldom carried out by investigators in the world. On the other hand, most reports concerning the haphazard use of mixed herbs against various human ailments have been emanated from the traditional Indian and Chinese ethno-medicine.

Therefore, ancient practitioners of this type of medicine claim that the medicinal plants have synergic effects (Sabu and Kuttan, 2002; Li, 2009). Moreover, the study of Javed and Akhtar (1990) revealed that methanol extract of combined Vernonia (V.) anthelmintica and Embelia (E.) ribes had reduced FECR% to (93) when administered to goats infested with GI nematodes. It should be explained that they didn’t assess each plant extract individually to determine its sole antinematicidal activity, thus, the impact of this combined extract is back chiefly to E. ribes and not to synergism with V. anthelmintica because the later plant

has a low antinematicidal potency (Iqbal *et al.,* 2006) as confirmed in the present study (Hamad, 2012). It could be mentioned that there is an absence of any type of synergy, additive and antagonism impacts between N. tabacum and A. indica when the pharmacological determinants for interaction of drugs are applied (Katzung, 2007). At the same time, the hypothesis of unavailability of any kind of drug interaction between trialed combined plants, which was reported by the author, may not be 100% accurate because such assumption requires a comprehensive study to be conducted on a vast number of medicinal plants. In this regard, we should remember that the kingdom of plant has a big diversity and each plant contains many various active ingredients, so presence of interaction between them could be expected.

7. Conclusions

AR is almost prevalent in every country particularly where sheep and goats are reared intensively. Resistance has developed by GI nematodes against the renowned broad-spectrum anthelmintics, especially among the benzimidazole/pro-benzimidazole group. Furthermore, AR is less common in remote rural areas where ethno-veterinary medicine is practiced through the use of ethnobotanical dewormers and access to allopathic antinematicidals is limited. It is noteworthy to mention that efforts to prevent rampancy of AR are ineffective because it has been rooted on all continents. It could be said that non-chemical alternatives such as, biological control, vaccination, genetic approaches, nutritional supplementation, and grazing management have not achieved a considerable result in the field. Alternatively, as a result of the futility of the aforementioned substitutes, phytomedicines are the suitable choice to control antinematicidal-resistant nematodes in small ruminants. In light of the studies that have been executed in different regions of the world, particularly in Asia, South Latin America and Africa, further phytopharmacological and phytotoxicological assessments are required when a given medicinal plant extract is trialed for its secondary impacts on the host and constructive influence on the parasite. Moreover, synergism between various active constituents within the same plant extract could be available and this, in turn, restricts the ability of parasitic nematodes to evolve resistance against herbal medicines. On the other hand, presence of synergy among ethnobotanicals remains controversial and, hence, more in vitro and in vivo studies are needed. Undoubtedly, trials for the evaluation of phytotherapeutical agents against antinematicidal-resistant nematodes are very rare; therefore, studies in this field should be elaborated by veterinary nematologists. Ultimately, more studies must be conducted in the field of pharmacognosy for this purpose.

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Conflict of interest statement

The authors of this review article attest no conflicts of interest regarding the information incorporated in this manuscript.

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Tables (n=3) in 3 pages

Table 1 Prevalence of antinematicidal resistance against different dewormers among gastrointestinal nematodes in small ruminants

1- Benzimidazoles	2- Imidazothiazoles	3- Tetrahydropyrimidines
(Most members)	(Levamisole HCL)	(Morantel and Pyrantel)
Very common	Common	Less common
4- Macrocyclic Lactones	**5- Salicylanilides**	**6- Amino acetonitrile derivatives**
(Ivermectin & Moxidectin)	(Closantel)	(Monepantel)
Common	Common	Perhaps not yet in field!!

Table 2 Geographical distribution of antinematicidal resistance among gastrointestinal nematodes of sheep in different countries (selected references)

Country	Antinematicidal drug	Reference (s)
Australia	BZ_s, LEV, Morantel, Closantel	Waller *et al.,* 1995
Brazil	BZ_s, LEV, ML	Echevarria *et al.,* 1996
Bulgaria	BZ_s	Iliev *et al.,* 2014
Denmark	BZ_s	Maingi *et al.,* 1996
France	BZ_s, LEV	Kerboeuf *et al.,* 1988 ; Palcy *et al.,* 2010
Germany	BZ_s, LEV	Bauer, 2001; Duwel *et al.,* 1987
Greece	BZ_s	Papadopoulos *et al.,* 2003; Gallidis *et al.,* 2011
India	BZ_s, Morantel, Closantel	Uppal *et al.,* 1992 ; Yadav *et al.,* 1993 Manikkavasagan *et al.,* 2015
Italy	LEV, ML	Traversa *et al.,* 2007
Kenya	BZ_s, ML, Closantel	Mwamachi *et al.,* 1995
Malaysia	BZ_s, LEV, ML, Closantel	Dorny *et al.,* 1994 ; Sivaraj *et al.,* 1994

Netherlands BZ$_s$, LEV, ML

Varady and Corba, 1999;

Borgsteede *et al.,* 2010

New Zealand ML

Hughes *et al.,* 2007

Pakistan BZ$_s$, LEV, ML

Hamad, 2012

Paraguay BZ$_s$, LEV, ML

Echevarria *et al.,* 1996

Scotland BZ$_s$, ML

Bartley *et al.,* 2003

Slovak Republic BZ$_s$, ML

Cernanska *et al.,* 2006

South Africa BZ$_s$, LEV, ML, Closantel Van Wyk *et al.,* 1999

Spain BZ$_s$, ML

Alvarez-Sanchez *et al.,* 2006 ;

Diez-Banos *et al.,* 2008

Sweden BZ$_s$, ML

Hoglund *et al.,* 2009

Switzerland BZ$_s$, ML

Scheuerle *et al.,* 2009

Trinidad BZ$_s$, LEV, ML

George *et al.,* 2011

United Kingdom BZ$_s$, ML

Taylor and Hunt, 1989;

Taylor *et al.,* 2009

U. S. of America BZ$_s$, LEV, ML

Miller and Baker, 1980;

Uhlinger *et al.,* 1992

Uruguay BZ$_s$, LEV, ML

Echevarria *et al.,* 1996

BZ$_s$: Benzimidazoles, LEV: Levamisole HCL, ML: Macrocyclic Lactones

Table 3 Assessment of antinematicidal activity of *Nicotiana tabacum* and *Azadirachta indica* extracts and their combination utilizing fecal egg count reduction [(FECR (%)] test, egg hatch assay (EHA) and adult motility test (AMT)

Plant's name	FECR (%)	EHA (LC$_{50}$ values)	AMT (LC$_{50}$ values) After 4 hours of exposure	Plant's rank (Potency)
N. tabacum	88.6	(0.566)* (0.496-0.642)**	(8.24)* (5.09-14.55)**	2
A. indica	85.14	(1.169)* (1.047-1.303)**	(15.40)* (9.99-26.05)**	3
N. tabacum & *A. indica* (Combined)	94.59	(0.523)* (0.465-0.586)**	(5.39)* (3.46-8.81)**	1

*= denotes LC$_{50}$ value of each plant extract
**= denotes 95% fiducial confidence interval (Lower-Upper)
N.: *Nicotiana* *A.*: *Azadirachta*

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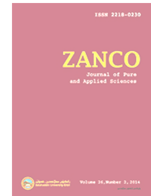

Antimicrobial susceptibility profile of aerobic bacteria collected from diabetic foot ulcer infections, In Sulaimani Province

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A B S T R A C T

This study was planned to determine the frequency and *in-vitro* susceptibility pattern of aerobic bacterial isolates from diabetic foot infections against different antimicrobial agents. A total of 62 isolated bacteria comprising of 44 (71%) Gram-positive bacteria and 18 (29%) Gram-negative bacteria were isolated from 50 specimens and the majority of infections were predominantly due to 38(76%) mono-bacterial and 12 (24%) mixed-bacterial. All bacterial isolates were tested for their *in-vitro* susceptibility to antibiotics using disc diffusion technique and broth method [(MIC) minimum inhibitory concentration]. Anti-microbial susceptibility results showed that vancomycin showed activity against Gram-positive bacteria while both gentamycin and ciprofloxacin had overall anti-microbial activity against *Pseudomonas aeruginosa* and *Escherichia coli*. Whereas *Serratia* spp. susceptible to tested antibiotic except for streptomycin. Broth macrodilution susceptibility testing demonstrated that 35/62 (56.4% %) aerobic bacterial isolates exhibited a MIC of 1.5g/mL for leaf extract of *Salix alba,* and 62/62 (100%) were susceptible with a MIC of ≤ 3 g/mL.

1- INTRODUCTION

Diabetic foot ulceration are complex, chronic wounds, which have a major long-term impact on the morbidity, mortality and quality of patients' lives and one of the main medical, communal, financial problems throughout the world (Islam *et al.*, 2013). They need urgent control of the infectious process by suitable debridement and antimicrobial drug use to prevent potentially devastating complications ([a]Lipsky *et al.*, 2012). Most lesions in formerly untreated patients (acute infections) are infected by aerobic Gram-positive cocci (frequently as monomicrobial infections) while chronic infections and previously treated

infections are often polymicrobial, usually with the addendum of aerobic or facultative anaerobic Gram-negative bacilli (Charles *et al.*, 2015). Antimicrobial resistance is one of our greatest serious public health threats (WHO, 2014). The most significant cause of antimicrobial resistance is overusing or misuse of antibiotics (Ventola, 2015). Patients having diabetic foot ulcer infections face more serious problems including multidrug resistant microorganisms (Trivedi *et al.*, 2014; [b] Lipsky *et al.*, 2012). The appearance and spread of multi-drug resistant strains with diabetic foot ulcer has made treatment much more complicated. Therefore, use of suitable antibiotics is necessary to avoid the risk of severity in foot infections of diabetic patients (Neto, *et al.*, 2012). It is important to be most familiar with the common pathogens to prescribe empiric antibiotics. *Salix* plants have long been used for treatment of diseases. *S. alba* is in family *salicacea*, commonly known as white willow. *S. alba* was described to contain many bioactive compounds which possess well antibacterial properties. Consequently, this study was designed with the objectives to determine the bacterial profile and to assess the *in vitro* antimicrobial susceptibility profile of bacterial isolates from patients with diabetic foot ulcer (DFU) to varieties of commonly used drugs and chloroform leaf extract of *Salix alba.*

2- Materials and methods

2.1. Ethics Statement

Ethical approval was granted from the Sulaimani teaching hospitals and the University of Sulaimani. Informed assent was obtained from each diabetic foot ulcer patient regarding demographic characteristics.

2.2. Study design

Total of 50 diabetic patients admitted in the Sulaimani teaching hospitals, Kurdistan region - Iraq, had the ulcer in their foot during September 2014 to October 2015 were included in this study.

2.3. Isolation and identification technique

All specimens were selected from new admitted diabetic foot ulcer patients, Figure 1. In order to avoid the isolation of normal microbial flora, debridement was performed to

clean the wounds and to obtain specimens by tissue biopsy, wound curettage, or aspiration techniques (Zubair *et al*.,2011). Gram staining was routinely done on specimens to get information about the types of organisms present. The specimen was inoculated onto appropriate aerobic planting media, such as blood agar, MacConkey agar and Nutrient agar and the plates were incubated overnight at 37°C. Any significant colony grown after 24h was isolated and identified according to morphological, cultural and conventional biochemicals (catalase, oxidase, and coagulase). All the strains were confirmed with the Vitek 2 Compact automated system by using Gram-Negative and positive (GN-and GP ID) cards according to the manufacturer's instructions (bioMerieux, Marcy l'Etoile, France).

2.4. Collection and extraction method of leaves of *Slix alba*

Leaf of *S. alba* collected between September and October 2015, at Topzawa-Dokan in the north of Iraq, because during this season leaves are almost completely developed (Szafranek *et al*., 2008). The healthy leaves were washed with demineralized water to remove the dust particles, dried, grinded and extracted with chloroform in Soxhlet equipment for 72 h, and then filtered by using whatman filter paper.

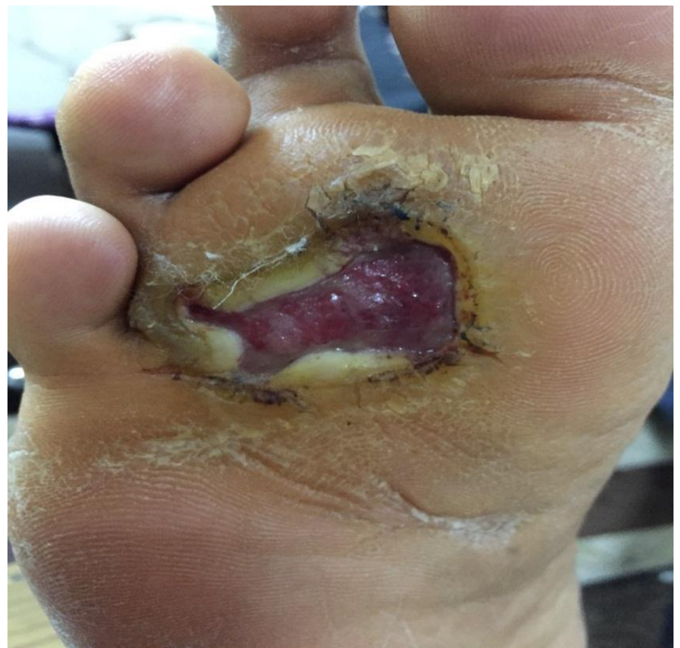

Figure 1: Neuropathic ulceration of the foot in a diabetic patient

2.5. Susceptibility testing

2.5.1. Disk diffusion method

Antimicrobial susceptibility testing of aerobic bacteria was performed using the disk diffusion method as recommended by the Clinical and Laboratory Standards institutes (Jeane B *et al*., 2015). Antimicrobial disks used were amikacin (30µg), amoxicillin (30µg), ampicillin (10µg), carbenicillin (100µg), cefuroxime (30µg), cefotaxime (30µg), gentamycin (30µg), erythromycin (15µg), tetracycline (30µg), penicillin (30µg), streptomycin (10µg), ciprofloxacin (30µg), rifampicin (30µg) and vancomycin (30µg). The 0.5 McFarland turbidity was prepared from pure colonies in brain heart infusion then streaked the swab over the entire surface of the Mueller Hinton agar plate. The antibiotic discs were applied with sterile forceps onto the surface medium. The inoculated agar plates were incubated at 37 ^{o}C for 24 h. The diameter of this zone was measured and compared with CLSI guidelines for zone sizes.

2.5.2. Determination of minimum inhibitory concentrations (MICs) of *Salix alba* leaf extract.

The MIC values of bacterial isolates were tested against extracted leaf. MICs were determined by using the broth macrodilution method (Szafranek *et al*., 2008). Briefly, 100 µL of adjusted bacterial suspensions equivalent to a 0.5 McFarland standard were added to a twofold serial dilution of extracted leaf that diluted in Mueller-Hinton broth. Based on the previously described method of Qureshi *et al*. (Qureshi *et al*., 2015), the antimicrobial activity of the extracts of *Salix alba* was studied in different concentrations (0.5, 1, 1.5 and 3 mg/mL) against six bacterial isolates. The results were observed after 24-hour incubation at 37°C. The minimum concentration of the extract inhibiting bacterial growth compared to the control culture was considered as the MIC. Duplicate tests were performed for each concentration.

3- Results

A total of 62 pathogenic bacteria were isolated from 50 specimens of patients with diabetes foot ulcer. The age of the patients ranged from 31-60 (mean age 50± 7) years, Table 1.

Table 1: Demographics of Diabetic Foot Patients

Gender	Age			Total	
	31-40	41-50	51-60	**Frequency**	**Percentage %**
Male	4	9	15	28	56
Female	5	6	11	22	44
Total	9	15	26	50	100

Gram-positive bacteria were the most frequently isolated pathogens 44 (71%) including *Staphylococcus aureus* 18 (40.9%), *Streptococcus pyogenes* 16 (36.3%), *Staphylococcus epidermidis* 8 (18.1%), and *Leuconostoc mesenteroides* 2 (4.5%). Gram-negative bacteria accounted for 18 (29%) of all bacterial isolates. *Pseudomonas aeruginosa* was the most frequently isolated Gram-negative 10 (55.5%), followed by *Escherichia coli* 6 (33.3) and *Serratia* spp. 2 (11.1%), Figure 2. In the current study, mono-bacterial were isolated from 38 (76%) patients and mixed-bacterial infections were notice in 12 (24%) patients.

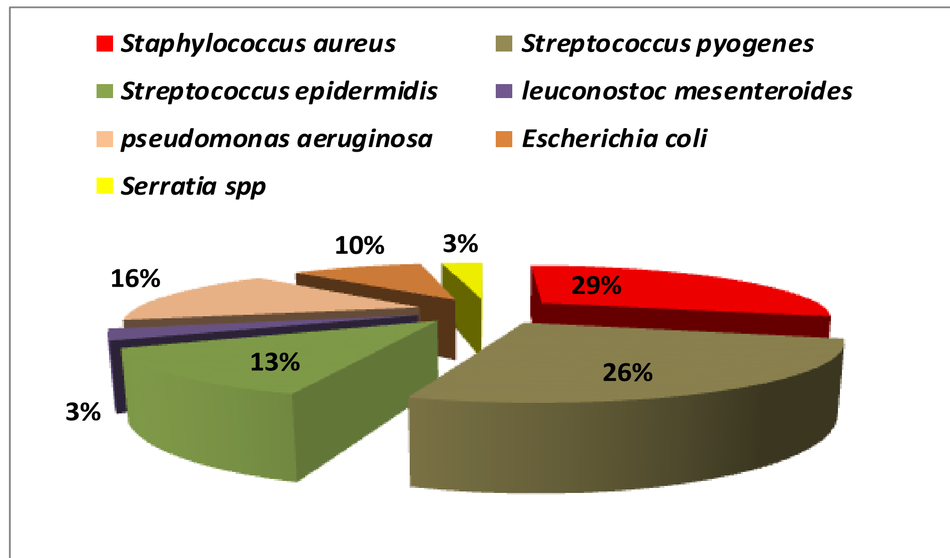

Figure 2: Frequency of bacteria isolated from diabetic foot patients.

Antibiotic resistance profile:

The results of antibiotics resistance studies are summarized in Table 2. Antimicrobial susceptibility results showed that Gram-positive bacteria were mainly resistant to rifampicin (63.6%), streptomycin (59%) and erythromycin (56.8.3%). The same rate of resistance (54.5%) was found to amikacin, penicillin, amoxicillin, ampicillin and tetracycline. Further, 47.7% of isolates were found susceptible to gentamycin, carbenicillin, cefuroxime, cefotaxime and ciprofloxacin. On the other hand, 93.2% of Gram-positive isolates were vancomycin-susceptible, the exception being 6.8%. Among the Gram-negative organisms, *P. aeruginosa* was resistant to most antimicrobial agents tested except for constant susceptibility to ciprofloxacin and gentamycin. *E. coli*, showed 100% sensitivity to vancomycin, gentamycin, ciprofloxacin and amikacin and 33% resistant to ampicillin, cefuroxime, and cefotaxime, while penicillin showed 83.3% resistance and 50% to the amoxicillin, carbenicillin, erythromycin and tetracycline. One *Serratia*

spp. isolate was susceptible to tested antibiotic except for natural resistance.

Table 2: Antimicrobial resistance pattern of gram-positive and gram-negative aerobes isolated in diabetic foot ulcer infections.

Antimicrobial Agents	*S. aureus* n=18	*S. pyogenes* n=16	*S. epidermidis* n= 8	*L. mesenteroides* n=2	*P.aeruginosa* n=10	*E.coli* n=6	*Serratia* spp. n=2	Total
Amikacin	15	0	8	1	7	0	0	31
Amoxicillin	15	0	8	1	7	3	0	34
Ampicillin	15	0	8	1	7	2	0	33
Carbenicillin	15	0	5	1	7	3	0	31
Cefuroxime	15	0	6	0	7	2	0	30
Cefotaxime	15	0	6	0	7	2	0	30
Gentamycin	15	0	6	0	0	0	0	21
Erythromycin	15	4	6	0	7	3	0	35
Tetracycline	15	0	8	1	7	3	0	34
Penicillin	15	0	8	1	7	5	0	36
Streptomycin	15	4	6	1	7	NT	1	34
Ciprofloxacin	15	0	5	1	0	0	0	21
Rifampicin	15	5	8	0	NT	NT	NT	28
Vancomycin	3	0	0	0	NT	0	NT	3

NT: Not tested

Leaf extract of *S. alba* susceptibility testing by the broth macrodilution method

In all experiments, positive controls without leaf extract yielded growth of the organism in 24hrs. Susceptibility testing demonstrated that 9/18 (50%) *S. aureus,* 9/16 (56.25%)

Streptococcus pyogenes, 6/8 (75%) *S. epidermidis*, 2/2 (100%) *L. mesenteroides,* 5/6 (83.3%) *E. coli* and 4/10 (40%) *P. aeruginosa* exhibited a MIC of 1.5 mg/mL for leaf extract. We observed that all bacterial isolates were susceptible to 3 mg/mL leaf extract, Figure 3.

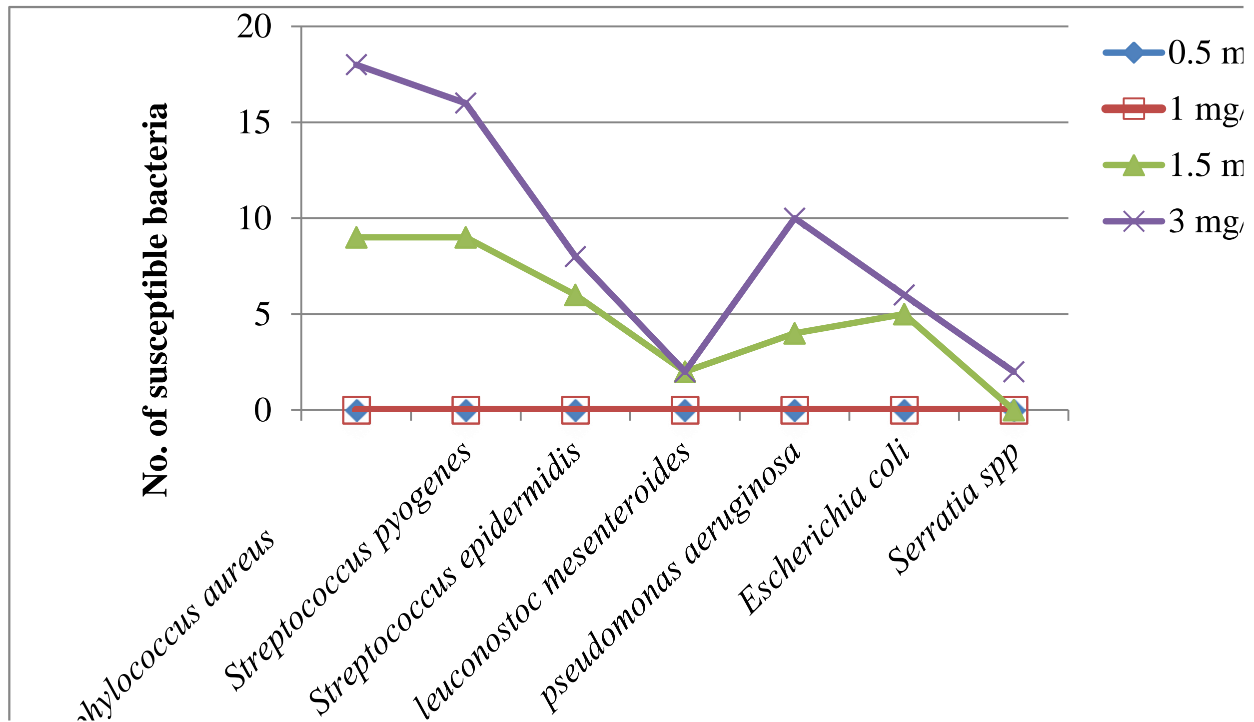

Figure 3: Result susceptiiblity testing of bacterial isolates to chloroform leaf extract of *S. alba*.

4- **Discussion**

With the increasing prevalence of diabetes mellitus, there is rising problem of infections, particularly foot ulcer infections. Diabetic foot ulcers may cause up to 20% of all diabetic hospital admissions (Perim *et al*., 2015). In our study mono-bacterial infections were reported in 76% foot infections while mixed-bacterial infections were seen only 24% cases. Several previous studies have reported the similar results (Shankar *et al*., 2005 and Murali, *et al*., 2014), whereas other studies have revealed contrasting results with polymicrobial infections being more predominant (Neto *et al*., 2012; Zubair *et al*., 2011). These discrepancies are due to differences in diabetic foot infections, severe diabetic foot infections regularly yield polymicrobial isolates, while mild infections are often monomicrobial (Citron *et al.,* 2007). We observed Gram-

ositive aerobic bacteria were most often isolated which is in accordance with the earlier studies (Zubair, *et al*., 2011; Perim *et al*., 2015). *S. aureus* and coagulase-negative staphylococci were the most frequently isolated bacteria and Gram-negative bacilli such as *P. aeruginosa, E. coli* and *Serratia* spp. These results are agreeable with the findings of other studies (Zubair *et al*., 2011; Szafranek, *et al*., 2008; Perim *et al*., 2015). Several studies have demonstrated that Gram-negative bacteria were isolated more frequently than Gram-positive bacteria (Raja 2007; Singh *et al*., 2009). These contrarieties could be partially due to differences in the causative agents occurring over time, geographical variations, or the kinds and severity of infection incorporated in the studies (Zubair *et al*., 2011). The *in-vitro* susceptibility data herein presented were interpreted as authentic, since the negative controls used in culture-based experiments remained negative. The antimicrobial susceptibility test showed vancomycin as the more active antibiotic against Gram-positive bacteria. These observations are important for patients' management. This result agrees with the results previously reported in a similar study conducted in Brazil on 89 bacterial isolates (Szafranek, *et al*., 2008). We found similar results that all *S. pyogenes* strains were susceptible to most antibiotics of the penicillin family, amikacin, ciprofloxacin and vancomycin (Singh *et al*., 2009; Rajalakshmi and Amsaveni, 2013). Gentamycin and ciprofloxacin were noticed to be the most effective antibiotics against gram-negative bacteria in our study. This is in accordance with the reports of Abdulraza *et al.* (2005). Our results showed that the majority of *P. aeruginosa* isolates were resistant to a large range of penicillin groups. These results were comparable to those achieved by Zubair *et al*. (2011). We found the *Serratia* spp. isolates to be susceptible to most of the tested antibiotics except amoxicillin, ampicillin, penicillin, and streptomycin. Kang *et al*. (2013) report similar finding from studies conducted in Korea (Kang *et al*., 2013). From a total of 62 bacterial isolates, 56.4% (26 Gram-positive bacteria and 9 Gram-negative bacteria) were susceptible to

leaf extract of *S. alba* with a MIC of 1.5 mg/mL whereas all isolates (100%) were susceptible with a MIC of ≤ 3 mg/mL. Chloroform leaf extract was found to be more active against Gram-positive bacteria (59%) than Gram-negative bacteria (50%), this may attribute to the differences in their cell wall structure. Gram-negative organisms are considered to be more resistant due to their outer membrane acting as a barrier to many environmental substances, including antibiotics. A similar finding has recently been reported by Tan *et al*. (2015), generally, ylang-ylang oil and different extracts of *Cananga odorata* demonstrated significant antibacterial activities against Gram-positive bacteria than Gram-negative bacteria (Tan *et al*., 2015). As such, our results contradict previous finding that Gram-positive bacteria are less susceptible than Gram-negative bacteria to different concentration of leaf extract (Parekh and Chanda, 2007). In conclusion, we have demonstrated that Gram-positive bacteria are the most common pathogens related with diabetic foot infections in Sulaimani. Those pathogens are regularly resistant to commonly used antimicrobial drugs, nevertheless remain susceptible to vancomycin. Gentamycin and ciprofloxacin have a good *in vitro* activity against Gram-negative bacteria. It was obviously observed that chloroform leaf extract of *S. alba* had good antibacterial activity against Gram-positive bacteria.

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