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# Activity of Honey and Propolis on Bacteria Isolated from Diabetic Foot

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Author HS designed the study, author BD performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors NS and MAG managed the analyses of the study. Author AA managed the literature searches. All authors read and approved the final manuscript.*

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## ABSTRACT

**Aims**: Diabetic foot ulcer is a significant complication of diabetes mellitus and often proceed lower extremely amputation. Propolis is a naturally occurring anti-inflammatory bee derived protectant resin. Previously, topically applied propolis has been reported to reduce inflammation and improves cutaneous ulcer healing in diabetic rodents. This study aimed to determine the Libyan honey and propolis activity and honey against bacteria isolated from diabetic foot ulcer lesion
**Study Design:** *In vitro* antimicrobial activities of honey and crude hexane and methanolic extract of Libyan propolis against bacteria isolated from diabetic foot ulcer lesion
**Place and Duration of Study:** Samples collected from patient in Tripoli Iben Nafees Hospital using disc and agar diffusion method.
**Methodology:** Disk diffusion method on groups of aerobic and anaerobic bacteria were obtained from diabetic foot lesion.
**Results:** The result showed that the percentage of aerobic bacteria isolated from diabetic lesion

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was about 74%, which include *MRSA, E. coli, Ps. aeruginose, Citrobacter, Pantoea, Proteus, Staphylococcus epidermis, Enterobacter and Serratia bacteria*. Whereas anaerobic bacteria reported about 26% of Clostridium, Bacteroides, and Lactobacillus Jensenii.

**Conclusion:** It was concluded that the honey and propolis extract had antibacterial activity against a different type of aerobic and anaerobic bacteria that were isolated from diabetic foot ulcer lesion.

*Keywords: Diabetic foot; Propolis; methicillin resistant Staphylococcus aureus; E. coli, Ps. aeruginosae; Citrobacter; Pantoea; Proteus; Staph. Epidermis.*

## 1. INTRODUCTION

Foot ulceration secondary to diabetes occurs in up to one quarter of people with diabetes [1]. They occur in people with type 1 and type 2, but usually arise much later after diagnosis in patient with type 1 diabetes, resulting from peripheral neuropathy, infection, ulceration and destruction of deep tissue these infection can develop in the skin, muscle, or bones of the foot as a result of the nerve damage and poor circulation that is associated with trauma or got deformity, lead to increase risk of gangrene [2]. Honey use as Topical honey has been used successfully in a comprehensive treatment of diabetic ulcers when the patient cannot use topical antibiotics [3].

Bees prepare honey by using nectar from flowers of different plants. Honey bees belong to genus *Apis*. Different varieties of honey are produced by different species of honey bees and these are collected by bee keepers. Honey is sugar rich as glucose 38.19%, fructose 21.28%, maltose and other sugars 8.81%, enzymes and pigments 2.21%, ash 1.0% and water 17.20% [4]. Honey can be used to overcome liver, cardiovascular and gastrointestinal problems [5]. Ancient Egyptians, Assyrians, Chinese, Greeks and Romans employed honey for wounds and diseases of the intestine. Since a few decades ago, honey was subjected to laboratory and clinical investigations by several research groups. The most remarkable discovery was the antibacterial activity of honey that has been mentioned in numerous studies [6]. Honey has low water content due to which most of the microorganisms do not grow in honey. In 1892, Van Ketel first recognised the antibacterial property of honey. Natural honey exhibits bactericidal activity against many organisms including *Salmonella, Shigella*, *Escherichia coli*, *Helicobacter pylori* [7,8].

Propolis is a resinous substance collected by worker bees (*Apis mellifera*) from the bark of trees and leaves of plants. This salivary and enzymatic secretions-enriched material is used by bees to cover hive walls to ensure a hospital-clean environment. As a natural honeybee hive product, propolis extracts have been used both internally and externally for thousands of years as a healing agent in traditional medicine. Propolis shows a complex chemical composition. Its biological properties- such as antibacterial, antiviral, antifungal, among other activities, have attracted the researchers' interest [9]. Previously, studies have shown broad-spectrum antimicrobial activity of various propolis extracts, antibacterial activity against *Enterococcus spp.*, *E. coli*, and *Staph. aureus* [10]. Also, researchers have reported that Libyan propolis have activity against *Trypanosoma brucei* and *Leishmania donovani* [11]. Therefore, in this study the antibacterial activity of Libyan Propolis and Honey on bacteria isolated from diabetic foot lesion of patient in Tripoli Iben Nafees hospital was determined.

## 2. MATERIALS AND METHODS

### 2.1 Reagents

Absolute ethanol, hexane, methanol, and Acrodisc syringe filters were obtained from Fisher Scientific (Loughborough, UK). Nutrient agar, MacConkey agar media, Mannitol salt agar Blood agar Cooked meat broth, DNase test, Augmentin, Metronidazol and Trptiase sugar agar were obtained from Oxod, England, UK. API 20E and API 20A were obtained from Biomerieux, France. Chromogenic was obtained from Liofilchem, Italy.

### 2.2 Bacterial Strains

Standard bacterial strains used in this study were *Escherichia coli* NCTC 12241/ATCC 25922*, Staphylococcus aureus* NCTC 12973/ATC*S.* The standard bacterial strains were activated and cloned three successive times in nutrient agar and stored on nutrient agar slants at 4℃. The identification of the local bacterial isolates was confirmed using conventional biochemical test [12].

## 2.3 Preparation of Propolis Extraction

Propolis and honey samples were collected from Tajora suburb in Libya. The beekeeper scraped the propolis sample off the top of the hive using a spatula and collected it in a clean tray. Propolis possessed an intense orange-like odour, was light brown and had a very sticky texture, and had a less intense odour. Crud propolis was dissolved in hexane solvent (nonpolar) for 4 days with mixing after that filtrated using Whatman NO1 filter paper. Filtrate solution was evaporated by rotator evaporation, and the solid extract was dissolved using methanol (polar), then repeat the same step when dissolve it in hexane. The crude solution extract was tested.

## 2.4 Collection, Isolation and Identification of the Bacteria Samples

This study has been made on 50 diabetic patients in Tripoli Iben Nafees hospital in the surgical department. All patients diagnosed as diabetic patient by the physician in the department. Data were collected from all patients using general questioner including history, name, age, sex, duration of diabetes mellitus, type of diabetes and natural of wound. Swabs were taken for bacteria culture, it cultured and the identification of bacteria carried out using Gram stain and API.

API 20 is standardised identification system for enterobacteriaceae and other Gram negative rods bacteria. This system that uses 23 miniaturised biochemical tests and a database. API 20 strip consist of 20 microtubes containing dehydrated substrate. Theses testes are inoculated with bacterial suspension which reconstitutes the media during incubation, metabolism produces colour changes that are either spontaneous or revealed by the addition of reagent. This test used for identification of aerobic and anaerobic bacteria. Small colony was taken from pure culture and then mixed with normal saline in test tube. The mixture added to microtube of API 20 (API 20 strip). The wool oil added to the word under of it line. The API 20 strip inculated for 24 hr to aerobic bacteria and 48 hr to anaerobic bacteria. Change in the colure of suspension indicates the type of bacteria.

The aerobic bacteria can be identified as the pus from the deep wound was taken by sterile swab transported to the cooked meat broth or nutrient broth and stored for 24 hrs befor cultivation. After 24 hours the swabs were cultured on MacConkey agar media, nutrient agar and mannitol salt agar. And then the culture was incubated for 18-24 hr(s). Staining different type of culture, from this test we can do API 20E for 18-24 hr(s) at 37℃. Some time to ensure from identification do: Oxidase test for non ferment, Indole test for *E. coli,* Capsule test for *Klebsiell*, chromogenic for *MRSA* and test for gram positive bacteria: coagulase test, DNAase test, API.

The Anaerobic bacteria can be identified by taken small quantities of pus from the deep wound by sterile swab was transported to the cooked meat broth or nutrient broth, heated at 100-180 on water bath for 10 minutes, then cooled suddenly to kill vegetative bacteria. Put the sample in anaerobic jar for 48 hrs at 37℃ to growth anaerobic bacteria, cultured the samples on blood agar, then put it again in anaerobic jar to isolated the pure bacteria for 48 hr(s) at 37℃. Routine test as gram stain can be used to identification of bacteria, then do API 20A, catalase.

## 2.5 *In vitro* Antibacterial Activities

Determine Antibiotic sensitivity test was carried out using disk diffusion method and using cup-cut diffusion method tested honey and propolis activity. Antimicrobial susceptibility was tested using paper disc agar diffusion method [13]. This method was performed using freshly prepared Mueller Hinton agar with overnight culture of bacteria inoculums, which were prepared by suspending the freshly grown bacteria in sterile normal saline and adjusted to a 0.5 McFarland standard. Paper discs (5 mm) were sterilised by autoclave and soaked in a propolis extracts (ethanolic and aquatic extract) solution with different concentrations (10, 20 and 30%). Solutions containing different propolis extracts solution at varying concentrations were placed separately in the plate under aseptic conditions. The agar plates maintained at room temperature for 2 h allowing for diffusion of the solution. All plates were then incubated at 37℃ for 24 h, and the zones inhibition were subsequently measured in millimeters [14]. The diameter of the zones of inhibition was measured. The inhibition zones were then measured in millimeters. Inhibition zones indicated a lack of microbial growth due to inhibitory concentrations. The antibiotics: Metronidazole, Augmentin (5 µg/ disk) was used as standards to compare the activity of honey in inhibiting the growth of bacteria. Each experiment was carried out three times.

## 2.6 Statistical Analysis of Data

The non-parametric data from the *in vitro* studies were analysed using a Mann Whitney U test for comparing two treatments or a a Kruskal Wallis test followed by Dunns ad hoc test for statistical differences between three or more treatments using the Statview® version 5.0.1 software package (SAS Institute Inc, Abacus Concept, *Inc.,* Berkeley, CA, USA). A p value of < 0.05 were considered significant compared to relevant control group.

## 3. RESULTS AND DISCUSSION

The bacteria collected from samples of diabetic patient foot ulcer lesion were isolated and identified. In this study the relationship between percentage of diabetic foot and sex was studied, the percentage of diabetic foot lesion in males was 72% and in females was 28% (Fig. 1).

The percentage of aerobic bacteria (74%) was higher than anaerobic bacteria (26%) from the collected lesion (Fig. 2). The aerobic bacteria include (Fig. 3); Methicillin-Resistant *Staph. aureus* (MRSA) at a highest percentage was 41% from total aerobic bacteria, then *Ps. aerginosa* (16%), *serratia* (15%), *Klebsiella* and *Citrobacter* (14%), *Staph. epidermis* (12%), *Enterobacter* (4%) where the *Proteus*, Non-fermenter, *Pantoea* and *E. coli* at *2%*. Anaerobic bacteria include (Fig. 4); *Clostridium* (54%), *Bacteroides* (41%) and *Lactobacillus* (5%).

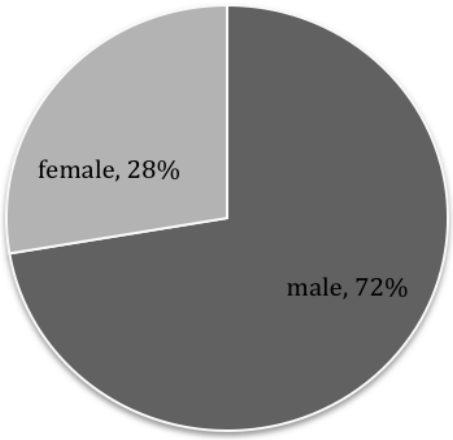


**Fig. 1. Shows the percentage of bacteria in diabetic foot lesion in males and females**

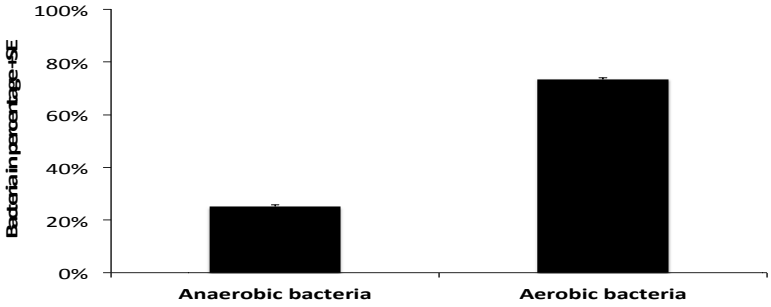


**Fig. 2. Shows the percentage of aerobic and anaerobic bacteria were obtained from diabetic foot lesion**

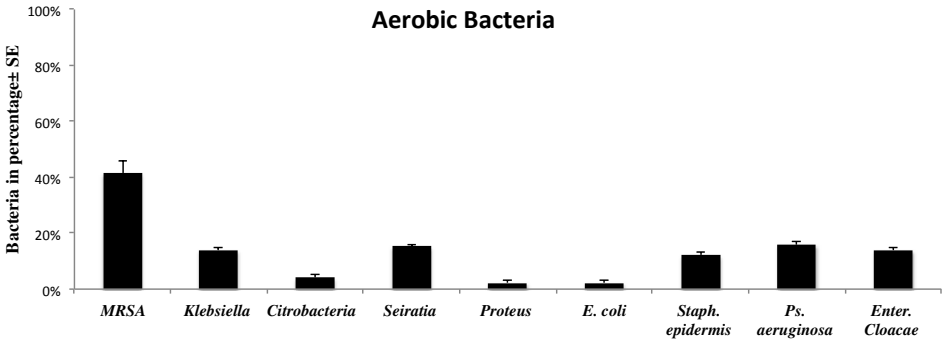


**Fig. 3. Shown type of anaerobic bacteria isolated from diabetic foot lesion**

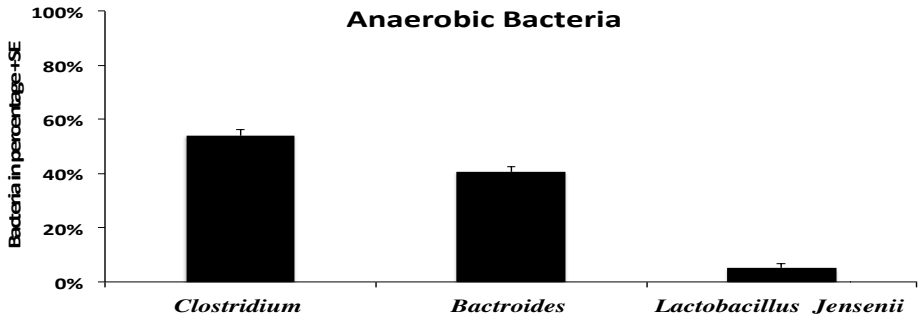


**Fig. 4. Shown type of aerobic bacteria isolated from diabetic foot lesion**

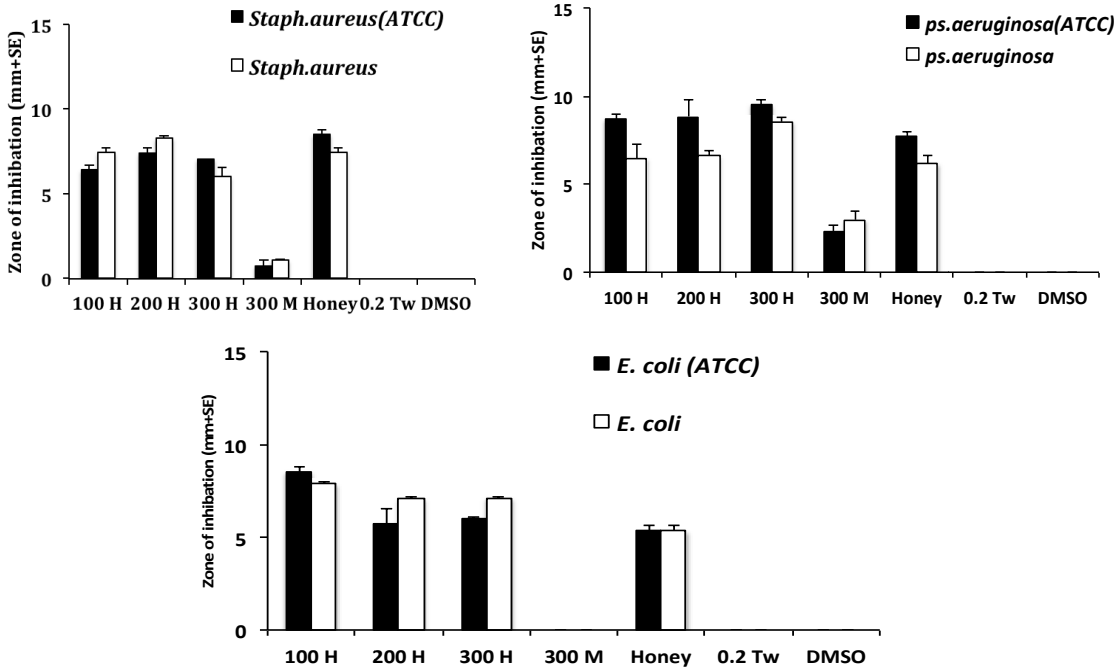


**Fig. 5. Comparison of data obtained to the results from the standard bacterial strains (ATCC)**

Foot ulcers are a very common complication of type 1 and type 2 diabetes individuals with diabetes have at least a 10-fold greater risk of being hospitalised for soft tissue and bone infections of the foot than the individuals without diabetes [15]. In this study male patients were more susceptible to bacteria foot infection than female diabetic patients. The previous studies have reported 'male sex' as a significant risk factor for non healing foot ulcers [16].

In present study the prevalence of aerobic bacteria was higher than anaerobic bacteria. This finding Compared with earlier reports, they recovered fewer anaerobic species [15]. In this study the aerobic bacteria include Methicillin-Resistant *Staph. aureus* (MRSA) at a highest percentage and *Ps. aerginosa* from total aerobic bacteria. Also the study bacteriological study of diabetic foot infection in Egypt has showed that 40% of diabetic foot infections were. *P. aeruginosa* and *S. aureus* [17]. Moreover, the microbial study for aerobic organisms from diabetic foot ulcers in India they reported that the most frequently isolated aerobic organisms were *Staph. aureus* and *Ps. aeruginosa* [18]. Also, an epidemiological survey of diabetic foot infections in Lisbon, reported that *Staph. aureus* was the most common cause of diabetic foot infections specially MRSA, were high and they concluded that probably related to previous indiscriminate antibiotic use[19] .

**Table 1. Explain the antibacterial activity of hexan propolis extract at 100 (100 H), 200 (200 H) and 300 (300 H) µg/ml, methanol propolis extract (300 M) and and honey measured by cup cut diffusion assay on groups of aerobic and anaerobic bacteria were obtained from diabetic foot lesion**

| Treatment / Bacteria | Zone of inhibition (mm ±SE) | | | | | | |
|---|---|---|---|---|---|---|---|
| | **100H** | **200H** | **300H** | **300M** | **Honey** | **MTZ** | **AMC** |
| *Staph. MRSA* | 5.3 ± 0.3 | 6.5 ±0.3 | 7.5±0.4 | 1 ±0.06 | 6.8 ± 0.2 | 0 ± 0 | 9.6 ±0.2 |
| *E.coli* | 5.1 ± 0.1 | 7.5 ± 0.2 | 8.3 ± 0.3 | 0 ± 0 | 8.1 ± 0.1 | 0 ± 0 | 9.7 ± 0.1 |
| *Non-fermenter* | 4.5 ± 0.3 | 5.2 ± 0.16 | 4.9 ± 0.03 | 4.9 ± 0.05 | 6.3 ± 0.2 | 7.1 ± 0.2 | 9.5 ± 0.3 |
| *Klebsiella* | 6.6 ± 0.3 | 5.9 ± 0.06 | 9.1 ± 0.2 | 0.74 ± 0.1 | 5.2 ± 0.1 | 1.63 ± 0.2 | 9.3 ± 0.3 |
| *Staph epidermis* | 7.4 ± 0.3 | 8.5 ± 0.3 | 5.5 ± 0.2 | 1.5 ± 0.2 | 7.2 ± 0.3 | 0 ± 0 | 9.8 ± 0.2 |
| *Proteus* | 6.1 ± 0.1 | 8.3 ± 0.3 | 7.1 ± 0.1 | 5.3 ± 0.3 | 5.3 ± 0.3 | 0 ± 0 | 0 ± 0 |
| *Citrobacter* | 3.6 ± 0.1 | 3.6 ± 0.1 | 5.6 ± 0.1 | 0 ± 0 | 9.6 ± 0.1 | 0 ± 0 | 7.6 ± 0.1 |
| *Enterobacter* | 3.9 ± 0.03 | 6.4 ± 0.3 | 7.3 ± 0.3 | 1.3 ± 0.3 | 9.0 ± 0.2 | 1.4 ± 0.3 | 9.5 ± 0.2 |
| *Ps. aeruginosa* | 5.43 ± 0.3 | 6.1 ± 0.3 | 8.4 ± 0.3 | 3.1 ± 0.3 | 6 ± 0.2 | 1.7 ± 0.14 | 10.2 ± 0.1 |
| *Serratia* | 5.5 ± 0.29 | 4.5 ± 0.4 | 2 ± 0.1 | 0.65 ± 0.1 | 5.5 ± 0.2 | 0.06 ± 0.0 | 8.2 ± 0.2 |
| *Bcteroides* | 5.6 ± 0.2 | 7.2 ±0.1 | 6.5 ± 0.2 | 1.2 ± 0.1 | 7.6 ± 0.3 | 2.2 ± 0.1 | 9.8 ± 0.4 |
| *Clostridium* | 8.4 ± 0.2 | 7.4 ± 0.3 | 7.5 ± 0.2 | 1.6 ± 0.3 | 8.4 ± 0.3 | 2.5 ± 0.2 | 9.8 ± 0.4 |
| *Lactobacillus* | 0 ± 0 | 5.5 ± 0.2 | 6.4 ± 0.2 | 3 ± 0.3 | 0 ± 0 | 0 ± 0 | 0 ± 0 |

*MTZ = metronidazole, AMC = augmantine*

The broad spectrum antimicrobial activity of honey has been demonstrated in various studies. Honey reportedly exerts both bacteriostatic and bactericidal activities. Because of the emergence of antibiotic-resistant microorganisms in diabetic wound treatment, the use of honey as an effective wound treatment is increasing because it can markedly inhibit the activities of wound-isolated microorganisms [20]. In this study the honey and Hexane extract of propolis had the antibacterial activity than methanolic extract. The study was to the antibacterial action of three different types of propolis extracts, water-extracted propolis, (propolis volatiles, and ethanol-extracted propolis were investigated by flow microcalorimetry coupled with polarography, and by Petri dish bioassay methods. The water-extracted propolis solution had the weakest antibacterial and antifungal action, compared to the other two extracts, which showed effects nearly similar to each other [21].

The Hexane extract and honey had the large zone of inhibition against most of tested bacteria isolated from food ulcer of diabetic patient except lactobacillus bacteria there was no inhibition by honey (0 ± 0.0), the zone of inhibition of propolis Hexane extract against lactobacillus was 6.4 ± 0.2m. The study was to evaluate the effect of ethanolic extract of Bulgarian propolis on 94 clinical anaerobic strains. The strains were tested by both agar-well diffusion (wells, 7 mm diameter) and disk-diffusion methods. Bulgarian propolis was active against most anaerobic strains of different genera. In addition to oral pathogens, an activity of propolis against *Clostridium*, *Bacteroides* and *Propionibacterium* species was observed [22]. It has been reported that decrease in wound odor during the treatment of diabetic foot and leg ulcers by using honey [23] Honey can exert its antimicrobial action both *in vivo* and *in vitro* against odour-producing bacteria, thus reducing their presence in wounds and consequently controlling malodor. Based on previous studies, honey can deodorise wound odour through two mechanisms. First, the presence of some anaerobic bacteria such as *Bacteroides* spp., *Peptostreptococcus* spp., and *Prevotella* spp. is documented to produce malodor. Second, wound odour is produced by the creation of amino acids through the decomposition of serum, tissue proteins, and dead cells by bacteria [20].

## 4. CONCLUSION

Libyan Propolis and honey presented the interesting antimicrobial activity. Honey was affective against aerobic and anaerobic bacteria isolated from diabetic foot. In this study, the Hexan was affective as a solvent in creating the antibacterial activity of the Propolis rather than methanol solvent. The differences and variations in the susceptibility to propolis between the Gram negative and Gram positive bacteria still remains an important subject for further investigations anaerobic bacteria.

## CONSENT

All authors declare that verbal informed consent was obtained from the paraticbate for publication of this study.

## ETHICAL APPROVAL

The study protocol was reviewed and approved by the Ethical Committees of National Authority for Scientific Research (NASR) of Libya in December 2012 by health ministry of Libya. All participants endorsed a written form informed consent.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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