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ROLE OF CAROB SEED IN CHARACTERIZATION OF MDR BACTERIA IN DIABETIC FOOT ULCER

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ABSTRACT : Diabetes mellitus is a noteworthy public health concern around the world. Foot ulcer, which is among the complications of diabetes are often the reason for diabetes-related hospital admission and may lead to the amputation of an infected foot if not immediately treated. The primary aim of this study was to estimate the role of carob seed extract as an active antimicrobial agent to treat multidrug resistance (MDR) bacteria isolated from diabetic feet and determine whether it has any toxicity to normal cells. Bacteria isolated from 57 patients with diabetic foot were identified, and their antibiotic sensitivity were determined. The aerobic bacterial isolates include *Staphylococcus aureus* (42.1%), *Pseudomonas aeruginosa* (28.95%), *Escherichia coli* (13.16%), *Enterococcus faecalis* (10.53%) and *Proteus mirabilis* (5.26%). Antibiotic susceptibility test was performed on 16 antibiotics and the results showed that 53.2% of the bacterial isolates had phenotypes with MDR. The ethanolic and aqueous carob seed extracts showed a wide spectrum of inhibitory effects against the MDR bacteria. Further, the extracts did not exhibit any cytotoxicity against normal Vero cell lines after 72 h of incubation. The carob seed extracts exerted inhibitory effects on MDR bacteria isolated from patients with diabetic foot and thus may be used in treating infection by MDR bacteria.

Key words : Antibacterial activity, carob seed, multidrug resistance, diabetic foot ulcer.

INTRODUCTION

Diabetes is a metabolic disease characterized by hyperglycemia, that outcomes from abnormal insulin activity, discharge, or both. Foot ulcer is a complication of uncontrolled diabetes. It can cause muscle decay, foot deformation, fringe neuropathy, and neuropathic crack. Diabetic foot ulcer shows as open ulcer in about 15% of diabetics and is usually situated at the bottom of the foot (Richard *et al*, 2011; Mendes *et al*, 2012).

Diabetic foot infection is complicated and generally polymicrobial in the bacteriology, involving aerobes and anaerobes. numerous investigators have noted different infection between aerobic and anaerobic pathogen (Chin, 2013). In fact, natural skin microbial flora is generally the first to pervade the fundamental tissue after the collapse of the innate defense process of the skin of the foot; then, different pathogens frequently synergize, causing a polymicrobial disease. It have been recognized various bacterial pathogens, extending from Gram-negative bacilli, like *Pseudomonas* species and Gram-positive cocci, such as *Staphylococcus aureus* (Lipsky *et al*, 2012).

Bacteria with multidrug resistance (MDR) are resistant to at least two antibiotics. MDR in hospitals and community environments are of significant concern to clinicians, patients, and pharmaceutical endeavors (Magiorakos *et al*, 2012). The widespread use of antibiotics and the length of time allotment at which medications are accessible in markets have presented serious issues, such as the rise of safe life forms abuse of antimicrobial drugs, overdosing, drug prescription with inappropriate susceptibility test, self- prescription and long-term hospitalization, which exacerbate MDR in developing countries (Nkang *et al*, 2009). Hospital-acquired infections due to MDR bacteria are considerably challenges for clinicians. *P. aeruginosa* and *S. aureus* are the most across the board MDR microorganisms (Godebo *et al*, 2013).

Owing to the high risk related to the utilization of synthetic antibiotics, medical plants and their extracts as herbal sources of natural antimicrobial agents against a wide scope of bacterial infections (Gram-positive and Gram-negative), including MDR species have attracted considerable interest (Kallen *et al*, 2010).

Medical plants produce secondary metabolites to protect themselves, the scientific enthusiasm in these metabolites these items are referred to by their action substances, for example, phenolics, terpenoids and alkaloids. These plants have developed compounds with significant therapeutic applications against human pathogens, including bacteria, fungi and viruses (Hsouna *et al*, 2015).

Ceratonia siliqua belongs to the legume (Fabaceae) family and is known as "carob" in Mediterranean regions. This plant with low-cost byproduct is exceptionally rich in important compounds. The crude extract of the tree has outstanding antioxidant capacity and potential medical benefits. The leaves, fruits and seed of this plant extract are usually utilize to manage a different of diseases and in addition as animal and human dite. The seed are also used in the production of gum often used in food products, especially ice cream, as a thickener (Hsouna *et al*, 2015; Roseiroa *et al*, 2013).

Interestingly, most researchers use normal Vero cell line to examine cytotoxicity because most drugs are toxic to normal cells and cause side effects. To the best of our knowledge, this subject has not been considered, so the aim of this study is to evaluate the activity of carob seed extract as an active antimicrobial agent to treat MDR bacteria isolated from diabetic feet and determine whether it has any toxicity to normal cells.

MATERIALS AND METHODS

From patients with diabetic feet admitted to the Diabetic Foot Center at Al Fayhaa General Hospital, a total of 57 clinical samples were collected after washing the ulcer with saline, specimens were collected by sterile swabs containing transport medium from deep and superficial surfaces, then immediately transferred to the laboratory.

Bacterial isolation

Five clinical bacterial strains were mostly isolated: *S. aureus*, *P. aeruginosa*, *Escherichia coli*, *Enterococcus faecalis* and *Proteus mirabilis.* They were processed in the laboratory and cultured in reasonable media. All samples cultured aerobically on MacConkey and blood agar plates for 24 h at 37°C. The phenotypic colony and cell were identified through Gram staining. Microscopic examination was performed by using the standard methods of the Bergey's Manual for Systematic Bacteriology for the identification of the recovered isolates. Conventional biochemical tests and Vitek®2 automated system were used to determine whether the isolated bacteria is Gram-negative or Gram-positive (Miller *et al*, 2018).

Antibacterial activity assays

The susceptibility of all bacterial isolates to various antibiotics was controlled through disc diffusion strategies, as prescribed by the Clinical and Laboratory Standard Institute. Commercial antimicrobial discs were used, and the isolates were cultivated and incubated at 37°C for 18 h at that point arranged a suspension utilizing split colonies for each isolate in 1–2 ml of normal saline. Every suspension was diluted with sterile normal saline to get a cell tally of around 10^6 CFU/ml utilizing standard turbidity (conforming to 0.5 McFarland tube) and 100 µl of each suspension was introduced to the focal point of the two well-dried MH agar plates and then spread homogeneously with a sterile cotton swab and left to dry for 15 min. A total of 16 different types of antibiotics for Gram positive and Gram negative bacteria were used for determining antibacterial sensitivity (Clinical and Laboratory Standards Institute, 2019; Coorevits *et al*, 2015).

Preparation concentrations of carob seed crude extract

Carob seed were bought from a herbal shop. The seed were washed with tap water, dried, then ground with a mixer. Then, the ground seed were extracted with sterile distilled water and absolute ethanol. For the biological activity test, 50 gram of carob seed extract (powder) was added to 500 ml of absolute ethanol after that an additional 50 grams was added to 500 ml of distilled water. The samples were preserved in a rotary shaker over night and filtered with no.1 Whatman filter paper and re-filtered through a 0.45 µm microfilter. The extracts were concentrated with a rotary evaporator at 50°C and stored at 4°C. Crude plant seed ethanolic and aqueous extracts with concentrations of 1000, 500, 250 and 125 mg/ml were prepared in dimethyl sulfoxide (DMSO) methanol (1:1 V/V) with notice that the eventual DMSO concentration was not above 0.1%.

Biological activity

All isolated bacterium of the bacterial suspension balance to 0.5 McFarland criterrion. Then, the suspension was diffusion on the whole roof of Muller Hinton agar with a sterile cotton swab. After drying, for each plate 9 mm diameter pore was made by utilizing cork borer in the company of repeat and control plates. About 0.1 ml of all carob extract concentration was applied to each well and incubated at 37°C for 24h. The diameter of inhibition zone was evaluated in mm (Bansode and Chavan, 2013).

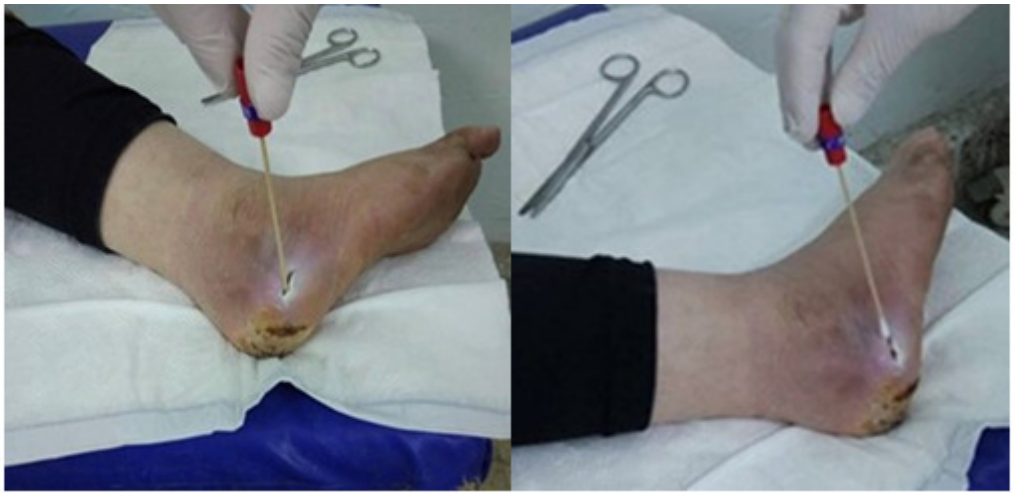

Fig. 1 : Swab taking from diabetic foot ulcer under sterile condition from deep and superficial surfaces.

Minimum Inhibitory Concentration (MIC)

The MIC was conducted to determine antibacterial activity. The MIC of carob seed extract was defined as the lowest carob seed extract concentration at which the visible bacterial growth was inhibited after 18 h of incubation at 37°C.

Cytotoxic activity

Cell cultures

Vero cell line tested in this assay was gained from the "Iraqi Center of Cancer and Medical Genetics Research (ICCMGR), Al-Mustansiryia University of Baghdad". The cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin 1% (v/v). In humidified atmosphere, the cells were inoculated in a 5% CO_2 and incubator at 37°C.

MTT Cytotoxicity assay and acridine orange/ propidium iodide staining

The cytotoxicity of the various concentrations of ethanolic carob seed extract was specified using MTT tetrazolium reduction technique, 10.00 mg/ml of a stock solution was gained by re-suspending the carob seed extract in DMSO (Sigma, St. Louis, MO). The sub-stock solution was set up through diluting the carob stock solution with the medium, and the eventual concentration of DMSO in whole the examinations didn't surpass 0.5% (v/v). The cells plated in 96-well plates in about 1×10^4 cells per well for 24 h and managed with various concentrations of carob seed extracts (400–1000 µg/ml). About 20 ml of MTT solution (5 µg/ ml in PBS) after 24, 48 and 72 h treatment added to each well, then incubated at 37°C for 4 h. All plates shook. The optical density was estimated at 570 nm with a microplate reader (Solanki *et al*, 2019). The method of cell passing incited by concentrate was inspected through AO/PI recoloring (Shahruzaman *et al*, 2019).

DNA Fragmentation assay

Vero cells were inoculated for 24 h in 25 cm of two-cell culture container previously to carob extract management, then treated with carob extract for 24 h then washed for PBS and purified with a DNeasy Blood DNA purification kit as well as Tissue Kit (Qiagen, Valencia, CA) according to the method of the manufacturer. The DNA was determined through electrophoresis with 1.5% agarose gel at 80–100 V, then the gel was pigmented using ethidium bromide, DNA was seen by a UV trans-illuminator.

RESULTS

Bacterial investigations

Of the 57 isolates, the most frequent bacterial isolates were *S. aureus* (42.1%), *P. aeruginosa* (28.95%) and *E. coli* (13.16%). Gram-positive bacteria were isolated more often than Gram-negative ones in the patients screened.

Antibacterial activity of selected commercial antibiotics

The antibiotic assay of the bacterial isolates to generally antibiotics used, gained using Kirby Bauer disk diffusion procedure which shown in Tables 1 and 2. Relating to the Clinical and Laboratory Standard Institute (CLSI) in the 16 commonly used antibiotics, Gram-positive *S. aureus* and *E. faecalis* showed resistance. Gram-negative *Enterobacteriaceae* family (*E. coli* and *P. mirabilis*) and non-fermenters *P. aeruginosa* were uniformly resistant to the tested antibiotics. Although some antibiotics were effective in inhibiting the growth of a bacterial type, they did not show any efficacy against another bacterial type. Interestingly, all Gram-positive

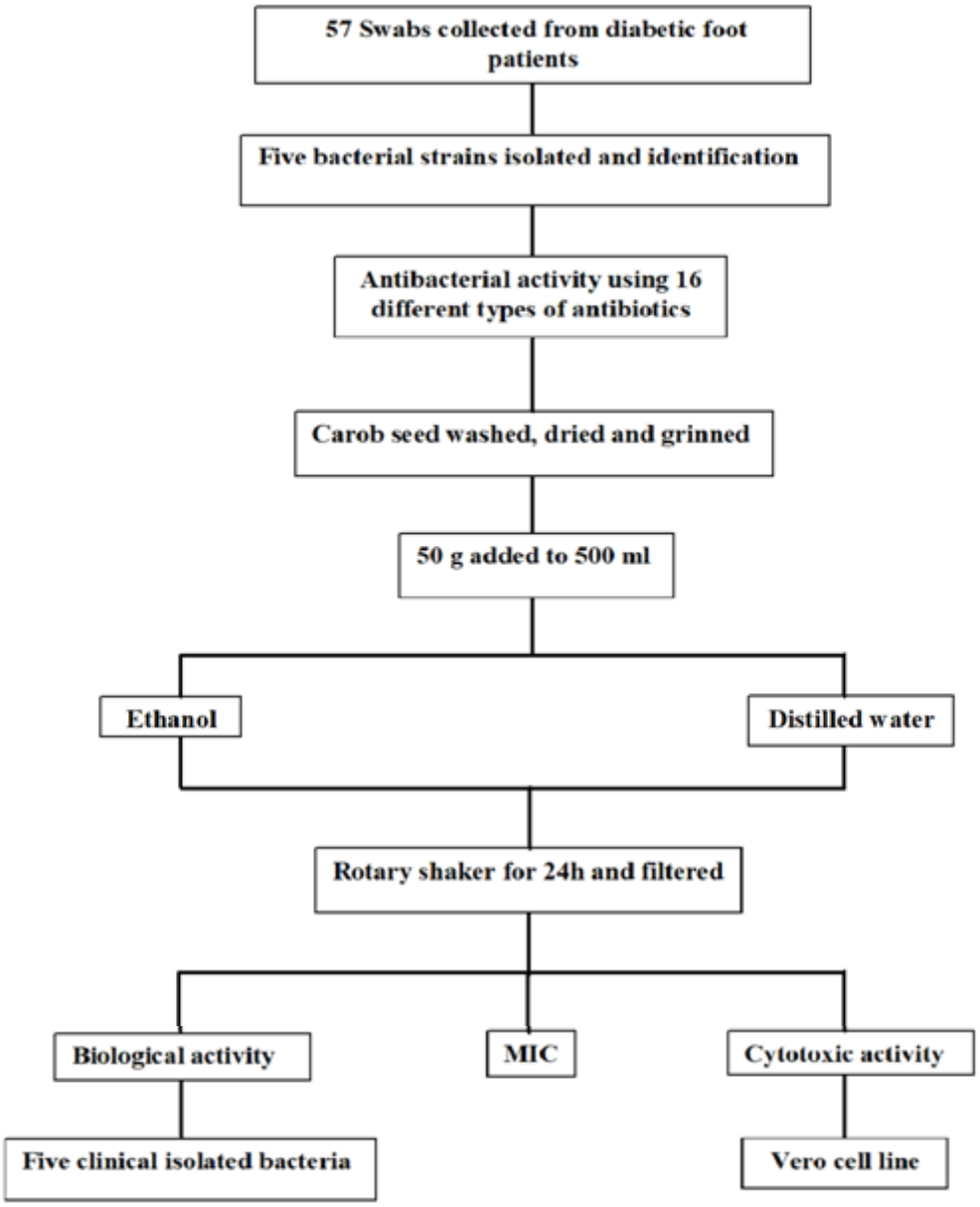

Fig. 2 : Shows a brief outline of the methods.

bacteria were sensitive to ciprofloxacin, gentamicin, nitrofurantoin and norfloxacin. Meanwhile, all the isolated Gram-negative bacteria were sensitive to cefepime and gentamicin. The antibiotic gentamycin showed intense activity against Gram-positive and Gram-negative bacteria.

Antibacterial activity of carob seed extract versus diabetic foot ulcer bacteria

The antibacterial activity (evaluated in terms of inhibition zone) of carob seed extracts was tested against five selected pathogenic bacteria. Table 3 and Fig. 2 showed the MIC results. In the agar diffusion assay, the results of the inhibition zone were observed around the tested materials and showed the strong antibacterial effect of carob seed extract against most tested bacteria. Notably, the ethanol extract was more active than aqueous extract, which had moderate activity against the tested bacteria. Despite the obvious effect of the aqueous and alcohol carob seed extract on Gram-negative and Gram-positive bacteria, carob extract showed stronger effect versus Gram positive bacteria than against negative bacteria. For ethanol and aqueous carob extract the concentrations used in MIC ranged from 50-500 µg/ml. At MIC of 100 µg/ml most of the bacteria were inhibited. These results are significantly important because of the small amount of carob seed extracts required to inhibit the growth of pathogenic bacteria.

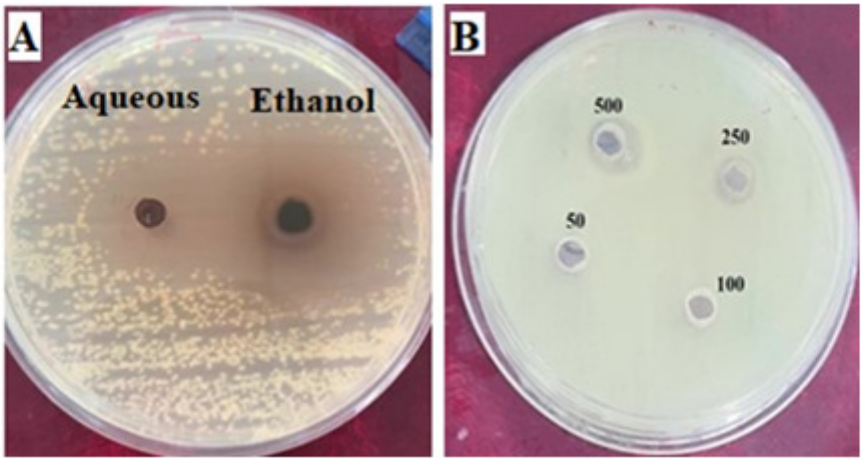

Fig. 3 : A- The diameters of the growth inhibitors of *Staphylococcus aureus* in ethanol and aqueous extract, **B-** The MIC of *Staphylococcus aureus* with the effect of the ethanol carob seed extracts.

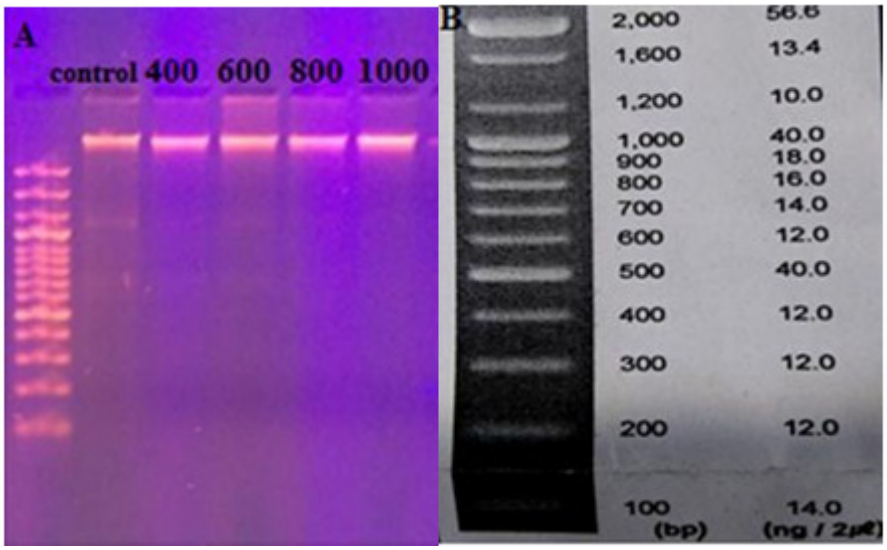

Fig. 4 : A- Genomic DNA separated of Vero cell line after 72 h managed with various concentrations ranged from 400, 600, 800 to 1000 mg/ml ethanolic carob seed extract. **B-** Range of the ladder applied 100-2000 (bp).

Table 1 : Antibiotic resistance patterns of Gram-positive bacteria.

Antibiotics	***Staphylococcus aureus***	***Enterococcus faecalis***
Ampicillin	R	S
Cefepime	R	R
Chloramphenicol	R	R
Ciprofloxacin	S	S
Erythromycin	S	R
Gentamycin	S	S
Levofloxacin	R	R
Linezolid	R	R
Nitrofurantoin	S	S
Norfloxacin	S	S
Ofloxacin	R	R
Penicillin	R	R
Rifampin	S	R
Tetracycline	S	R
Trimethoprim-Sulfamethoxazole	S	R
Vancomycin	R	S

*R resistant *S sensitive.

Table 2 : Antibiotic resistance patterns of Gram-negative bacteria.

Antibiotics	***Pseudomonas aeruginosa***	***E. coli***	***Proteus mirabilis***
Amikacin	S	R	R
Aztreonam	R	R	S
Cefepime	S	S	S
Ceftoaxim	R	R	R
Ceftriaxone	R	R	R
Ciprofloxacin	R	S	S
Gentamycin	S	S	S
Imipenem	R	R	S
Levofloxacin	S	R	S
Nitrofurantoin	R	S	S
Ofloxacin	R	S	R
Piperacillin	R	R	R
Piperacillin/Tazobactam	R	S	R
Tetracycline	S	S	R
Tobramycin	S	R	S
Trimethoprim-Sulfamethoxazole	R	R	R

*R resistant *S sensitive.

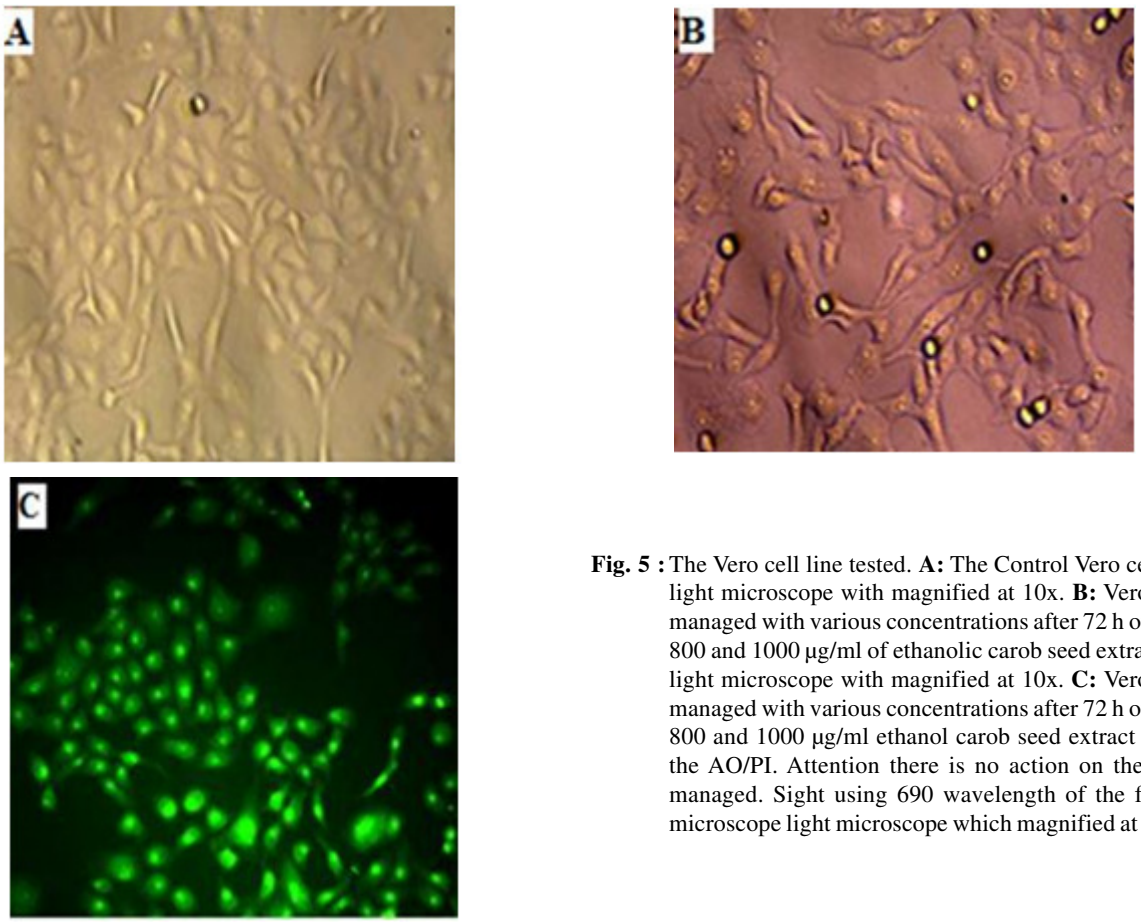

Fig. 5 : The Vero cell line tested. **A:** The Control Vero cell beneath light microscope with magnified at 10x. **B:** Vero cell after managed with various concentrations after 72 h of 400, 600, 800 and 1000 µg/ml of ethanolic carob seed extract beneath light microscope with magnified at 10x. **C:** Vero cell after managed with various concentrations after 72 h of 400, 600, 800 and 1000 µg/ml ethanol carob seed extract stained by the AO/PI. Attention there is no action on the cell after managed. Sight using 690 wavelength of the fluorescent microscope light microscope which magnified at 10x.

Table 3 : The antimicrobial effectiveness of carob seed extract against diabetic foot bacteria and the definition of the minimal inhibition concentration (MIC) µg/ml.

Bacterial strains	No. of isolated bacteria from 57 samples (%)	Inhibition zone (mm)			
		Ethanol extract		Aqueous extract	
		Inhibition zone	MIC	Inhibition zone	MIC
Gram positive bacteria					
Staphylococcus aureus	16 (42.1%)	28	100	20	250
Enterococcus faecalis	4 (10.53%)	20	250	17	100
Gram negative bacteria					
Pseudomonas aeruginosa	11 (28.95%)	24	100	19	100
Escherichia coli	5 (13.16%)	18	100	15	50
Proteus mirabilis	2 (5.26%)	16	50	14	50

Cytotoxicity assessment

Cytotoxicity of the ethanol carob seed extract on the Vero cell line. The examined concentrations to the cell line were ranged 400, 600, 800, and 1000 µg/ml in 72 h incubation. The MTT test detected that ethanol carob seed extract showed no cytotoxic effect against the Vero cell line at all concentrations.

The genomic DNA and staining with ethidium bromide in agarose gel isolated from Vero cell line after processing with carob seed extract showed no evident of DNA fragmentation confirmed (Fig. 3) and in relation to AO/PI coloring, no any death of cell line occurs after the processing (Fig. 4).

DISCUSSION

Foot ulcer in diabetic patients is a serious and difficult health problem for medical staff. Furthermore, the complication of the disease may be due to the multiplicity of the types of bacteria associated with the ulcer. In the present study the most generic pathogens isolated were Gram-positive bacteria. In spite of the results of this study are compatible with the results of former surveys that showed the dominance of Gram-positive bacteria in

diabetic foot ulcer (Zubair *et al*, 2010; Ozer *et al*, 2010), another studies notified that Gram-negative bacteria were dominant in specific areas (Pappu *et al*, 2011; Shanmugam *et al*, 2013). The difference in results is possibly due to the difference in sampling type (deep wound swab or tissue sample).

In this study antibiotics sensitivity tests revealed that all the bacterial isolates for Gram-positive and Gram-negative bacteria showed MDR to commonly used antibiotics applied to diabetic foot ulcer. The presence of these MDR bacteria may be consequent to the utilize of broad spectrum antibiotics. Furthermore, patients with diabetic foot ulcers are commonly hospitalized many times and generally risky to various courses of antibiotics, that may have led to antibiotic resistance. These findings agree with many studies about the presence of MDR bacteria in diabetic foot ulcers (Perimet *et al*, 2015; Singh *et al*, 2018). In the present study, gentamicin was the most active antibiotic of all the diabetic foot ulcer bacterial isolates and is compatible with the findings of a former study (Hariharan *et al*, 2015).

The outcome of the present study clearly showed that ethanolic and aqueous carob seed extract act as antibacterial agents against the ûve tested bacterial species. However, most previous studies were performed on carob tree and did not use the seed extracts. Thus, this study is the first to investigate the activity of carob seed extracts against diabetic foot ulcer associated with MDR bacteria.

The efficiency of the active compounds in the carob seed extracts showed areas with growth suppression. These areas were presented by clear areas surrounding the well. The susceptibility of the bacterial species isolated from diabetic foot ulcer, especially *S. aureus* and *P. aeruginosa*, to the employed carob seed extracts were significant because infections by these types of pathogenic bacteria have become a genuine concern for hospitals, particularly with regard to fundamentally sick and immunocompromised patients (Amenu, 2014; Bassetti *et al*, 2018). The antibacterial activity of the carob seed extracts might be due to their active components, such as polyphenolic compounds, which are responsible for most of the extracts' medicinal action (Azab, 2017).

The investigation for novel therapeutic drugs from natural plant products for the treatment of diabetic foot ulcer is based on the cytotoxic properties of natural samples. Thus, normal the Vero cell line was used in the testing of the cytotoxicity of carob seed extract in the present study. The results indicated that all the seed concentrations did not present cytotoxicity and did not lead to DNA fragmentation or apoptosis and necrosis. However, despite the effectiveness of the carob seed extract in reducing the growth of resistant bacteria associated with diabetic foot ulcer, carob plant must be further investigated with regard to its applications in pharmaceutics and potential use as a disinfectant.

CONCLUSION

Ethanol and aqueous carob seed extract have great potential as antibacterial compounds against MDR bacteria associated with diabetic foot ulcer as compared with commercial antibiotics. Therefore, these natural products can be used in the treatment of foot ulcer.

Conflict of interest

The authors declare no conûicts of interest.

Consent for publication

Not applied.

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