Antibacterial efficiency of natural products against multiple-drug-resistant clinical isolates

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Background
Antibiotic resistance is a global problem that has aggravated recently to threaten humans, cattle, and crops. This has inspired scientists to examine various natural products, herbs, and plants that have been used since antiquity for their valuable medicinal potential. They have not only proven less likelihood to produce resistant strains but also exert a positive effect on beneficial probiotics boosting the general health status of the host.
Objective
To identify the major multiple-drug-resistant bacteria underlying diabetic foot ulcer infections and screen and select herbs and natural extracts, commonly available in local herbal stores, for their activity against the isolated bacteria.
Material and methods
Bacteria isolated from diabetic foot ulcers of hospitalized patients were identified according to their morphological and biochemical properties. The isolated strains were tested against extracts of bitter melon, honey, pomegranate peel, myrrh gum, and turmeric powder using the *in vitro* agar well-diffusion assay technique.
Results and conclusion
The bacterial isolates were resistant to all of the tested standard antibiotics and identified to belong to five different genera: Gram positive bacteria *Staphylococcus aureus* and *Streptococcus pyogenes* and Gram negative bacteria *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. All of the natural preparations exerted different levels of antibacterial activity except for bitter melon. These findings shed tremendous light on the up-till-now promising effect of the natural antibiotics arsenal and necessitate the importance of systemically studying their individual and synergistic mechanisms, interactions, and kinetics.

Keywords:
antibiotic resistance, bitter melon, complementary and alternative medicine, curcumin, honey, myrrh gum, pomegranate peel, turmeric

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Introduction

Infectious diseases are a major cause of morbidity and mortality, especially in developing countries. Furthermore, resistance to antibiotics is a serious global health problem that jeopardizes patients' prognosis and adds to the load on the health care system and therapy costs, as the treatment time is prolonged and second or third line of antibiotics is used [1]. Bacteria will inevitably keep developing ways to change relevant features to survive exposure to new antibiotics via mutations or DNA exchange (horizontal gene transfer), resulting in what is commonly called 'superbugs.' This usually describes when the microorganism is resistant to two or more different antibiotic classes [2]. Gram-positive bacteria are less prone to antibiotic resistance on account of the porosity of the thick peptidoglycan cell wall that characterizes them, in comparison to the more intact outer membrane of the Gram negative bacteria [3]. Antibiotic resistance arises from many reasons, including (i) misuse of antibiotics (prolonged use, self-medication, inappropriate or indiscriminate prescription, wrong doses, discontinued treatment, etc.), (ii) use in cattle for enhancing growth, (iii) using contaminated water in the soil or for potable use, and (iv) population mobility and traveling over large distances [1,2].

There are several mechanisms by which microorganisms develop resistance. These involve degradation, modification, or expulsion of the antibiotic or changing their own antibiotic targets, uptake rate, or antibiotic-affected cellular processes. (a) Antibiotic degradation is exemplified by β-lactamases that hydrolyze penicillins and cephalosporins, hydrolyzation of tetracyclines by TetX, and phosphorylation or hydrolysis of

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macrolides [3,4]. (b) Drug modification is depicted by the addition of acetyl group to aminoglycosides, chloramphenicol, streptogramins, and fluoroquinolones and phosphate or adenyl groups to aminoglycosides. (c) Efflux pumps expel antibiotics using ATP, in the case of the ABC family, or according to the ion gradients, in the cases of MFS, RND, MATE, and SMR proteins. (d) Changing the target sites includes the following mechanisms: (i) decreasing the affinity of penicillin-binding proteins (PBP) classes A and B in Gram positive bacteria, hence lowering susceptibility to β-lactam drugs; (ii) glycopeptide vancomycin resistance via replacing the target D-Ala-D-Ala of the peptidoglycan cell wall with the less affinitive D-Ala-D-lac or D-Ala-D-Ser by the action of *vanHAX* cluster; (iii) changing the structure and charge of the cell membrane, inhibiting the binding of calcium and consequently, lipopeptide daptomycin through mutations in genes (e.g. *mprF*); (iv) ribosomal mutations (e.g. L4 or L22 proteins in 23S rRNA), methylation (e.g., via *erm* genes), or protection leading to aminoglycosides, oxazolidinones, macrolides, and tetracycline resistance, (v) modifications in DNA gyrase (e.g. *gyrA* gene) or topoisomerase IV (e.g. *grlA* gene), leading to resistance to the antibiotics aminocoumarins and fluoroquinolones that attack nucleic acid synthesis. (e) Restricting the antibiotic uptake is shown in vancomycin-intermediate and resistant *Staphylococcus aureus* (VISA and VRSA) by developing a thicker cell wall and retaining the vancomycin outside the cells [3,5,6]. Another example is when the *Enterobacteriaceae* decrease the number and affinity of porin cell membrane channels (Omp C or Omp F), leading to carbapenem resistance. Biofilm formation is also considered another case as the biofilm matrix constitutes a natural barrier against antibiotic penetration. (f) Methicillin-resistant *S. aureus* can incorporate an exogenous β-lactam-resistant PBP, known as PBP_{2a} and encoded by *the mecA* gene, into the growing peptidoglycan cell wall bypassing the β-lactam drug's activity [4–6].

Many patients prefer complementary and alternative medicine (e.g. acupuncture, Ayurveda, chiropractic treatment, and natural and herbal remedies) over conventional pharmaceutical drug therapy. In this respect, there are many related reasons and factors like (i) the kind and severity of the illness; (ii) availability of professional health care; and (iii) patients' education, socioeconomic level, ideologies, and cultural beliefs [7,8]. The common perception of their safety, being free of adverse effects, low cost, and easy access through healthy food or herbal shops, increases the likelihood of self-prescription and nonexpert recommendation [8]. However, it is unfair to state that the value of herbal medicine or ethnobotanicals stems only from folkloric practices. Natural products are still considered an important and continuous mine for discovering various bioactive compounds, and many of these compounds, or analogs thereof, have found their way to standardized drug formulations to alleviate different diseases [7,9]. Examples are many and diverse concerning structure and origin, including, but not limited to, the cardiac glycoside stimulant digoxin from *Digitalis purpurea*, the aspirin precursor salicylic acid derived from *Salix* spp. willow bark, the antihypertensive alkaloid reserpine from *Rauwolfia* spp., the cough suppressant codeine from the seed capsule of *Papaver somniferum*, the antimalarial quinine from *Cinchona* bark and arteether, the derivative of artemisinin from *Artemisia annua* plant, and the cytotoxic drugs paclitaxel, isolated from the Pacific yew tree *Taxus brevifolia*, and camptothecin, from the deciduous tree *Camptotheca acuminata* [7,9].

In this report, we evaluate the in vitro activity of bitter melon, honey, myrrh gum, pomegranate peel, and turmeric powder against selected pathogenic antibiotic-resistant Gram-positive and Gram-negative clinical isolates.

Material and methods

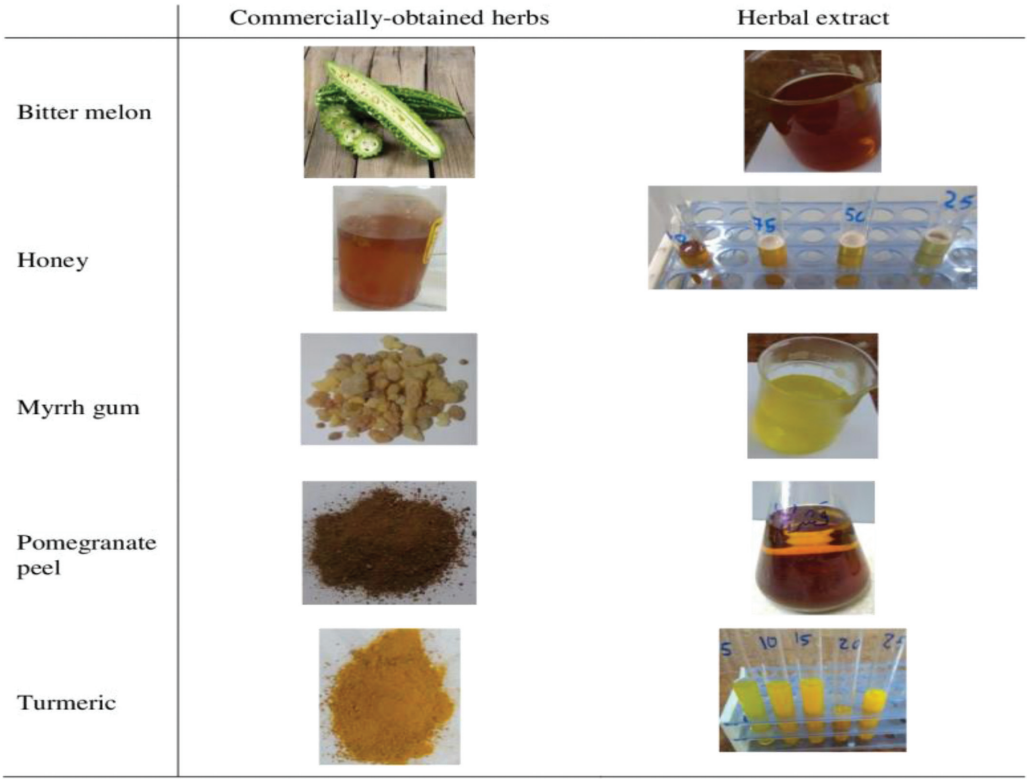
All chemicals used here were of analytical grade and purchased from Sigma-Aldrich (St. Louis, Missouri, United States) unless otherwise stated and were used as received without further purification. The microorganisms were obtained from a clinical laboratory sample collection, without direct contact with the concerned patients. All solutions were prepared using deionized water of resistivity not less than 18.2 MΩcm.

Preparation of the tested samples

All test participants, shown in Fig. 1, were obtained commercially from local grocery and herbal stores.

Bitter melon or bitter gourd (*Momordica charantia*): dry bitter melon was thoroughly ground and homogenized using mortar and pestle. Overall, 100 g of powder was wrapped in Whatman grade 1 filter paper and extracted with ethanol in a Soxhlet extractor tube until complete extrication was achieved. The extract was then dried under vacuum using a rotary evaporator (Hei-VAP Precision, Heidolph, Schwabach, Germany) and stored at 20°C until further use [10].

Figure 1



Natural products and herbs tested against multidrug-resistant clinical bacterial isolates.

Honey samples were first filtered using sterile mesh/gauze to remove debris and then streaked on blood agar to verify sterility and stored at 2–8°C until used as serial aqueous dilutions of 25, 50, 75, and 100% (v/v) [11].

Myrrh gum (*Commiphora molmol*): 2 g of myrrh resin was ground into fine pieces using mortar and pestle and soaked in 5 ml of 95% ethanol, decanted, and centrifuged at 12×10^3 g for 10 min. The combined supernatant was then dried under vacuum using a rotary evaporator and kept in the least amount of ethanol until further use [12]. As the concentrated oil is insoluble in water, 10% (v/v) solution [equivalent to 7.5% (w/v)] of the oil in ethanol was used as a stock solution to obtain the experimental concentrations, that is, 0.25, 0.5, 1, 2, and 2.5 (w/v).

Pomegranate peel was obtained from fresh mature fruits of *Punica granatum* L. The peel was removed and dried for 10 days and then thoroughly ground and homogenized. Overall, 50 g of the peel powder was extracted using absolute methanol. The extract was then centrifuged at 8000 rpm for 15 min (Z 326 K, HERMLE Labortechnik GmbH, Wehingen, Germany) and then the supernatant was dried under vacuum using a rotary evaporator (Hei-VAP Precision, Heidolph) and kept at 20°C until further use where serial dilutions were prepared in dimethyl sulfoxide [13].

Turmeric powder (*Curcuma longa*) was used as the commercially available pulverized roots, in serial aqueous dilutions of 5, 10, 15, 20, and 25 g%.

Test organisms

The tested microorganisms were kindly procured from a private medical laboratory, being isolated from patients with diabetic foot ulcers. The identification of bacterial isolates was done according to Bergey's manual of systematic bacteriology, criteria set by the Clinical Laboratory Standard Institute (CLSI), and several other basic practical biochemical guides [14–16] and verified to be multiple-antibiotic-resistant. The organisms were maintained and subcultured on nutrient agar.

Antibiotic susceptibility test

The test was carried out per the Kirby-Bauer method [17]. Inocula were adjusted via the suspended turbidity to 0.5 McFarland standards corresponding to OD_{600} of 10^6 colony-forming units/ml, using a UV/Vis spectrophotometer (Jasco V-630, Tokyo, Japan), and incubated in nutrient broth for 24 h in an incubator shaker at 150 rpm and 37°C. These cultures were later

used to inoculate the nutrient agar plates within 15 min of standardizing the inoculum to avoid changes in the inoculum density. Wells of 9-mm diameter were punched into the agar plates using a sterile cork-borer and filled, under aseptic conditions, with 50 µl of the test samples of turmeric powder, honey, and myrrh gum. The same volume of bitter melon and pomegranate peel extracts were loaded onto 5-mm diameter discs. Dimethyl sulfoxide was used as a negative control. The plates were allowed to stand for 1 h to allow for prediffusion of the samples into the medium. The plates were pre-diffusion incubated aerobically in an upright position at 37°C for 24 h. The antibacterial activity was evaluated by measuring the zone of inhibition (mm). Standard antibiotic susceptibility testing discs from Bioanalyse (Ankara, Turkey) were used to confirm the antibacterial scope of the tested organisms. The discs were 5 mm in diameter and the tested antibiotics were the first-generation cephalosporin cephradine (20 µg), macrolide erythromycin (15 µg), aminocoumarin novobiocin (30 µg), β-lactam antibiotics oxacillin (1 µg) and penicillin G (10 U), aminoglycoside tobramycin (10 µg), and the glycopeptide vancomycin (30 µg). This experiment was performed in triplicate. The data were expressed as mean±SD.

Results and discussion

The skin is the largest organ in the human body and is considered the first line of defense against different environmental conditions and pathogens. Additionally, it exerts significant regulatory and sensory roles. Therefore, proper wound treatment is pivotal for maintaining homeostasis and ensuring a person's general health and well-being.

Diabetes mellitus is an impairing and debilitating disease that affects many organs including the skin. Almost half the diabetic patients, with type 1 or 2, develop one or more dermatologic complications during their disease [18]. Skin diseases are clinically scrutinized to detect any metabolic discrepancies and track the glycemic control of the case. Diabetic foot syndrome is among the common cutaneous implications and is responsible for a high level of morbidity, hospitalization, and mortality. It leads to loss of skin integrity and underlying vasculopathy rendering the site susceptible to infection. This is aggravated, as well, by the repressed immune system and impaired phagocytic capacity [2,18].

The involved bacterial strains, reported in this study, were isolated as a part of a previous investigation and identified here via basic biochemical analyses [14–16]. Hence, samples were plated on nutrient agar, MacConkey salt agar, mannitol salt agar, and blood agar. The media were checked for the formation of clear colonies whose morphology was also noted. Additionally non-lactose lactose-fermenting colonies and nonlactose fermenting colonies were described by pink color and pale color, respectively, on MacConkey agar. Small colonies surrounded by yellow zones or colonies changing the color of mannitol salt agar to yellow and white creamy colonies were also observed. The isolates were Gram stained in addition to other biochemical and enzymatic tests, namely, oxidase, catalase, indole, motility, citrate, urease, and triple sugar iron agar tests, as shown in Table 1. Despite not being completely inclusive, the bacterial isolates identified in Table 1 were the most dominant genera depicted in other investigations as well. It can be also noted that Gram-negative bacteria represented 60% of the strains. This accords with the classification of drug-resistant bacteria isolated from infected wounds in different clinical facilities, most relevantly from diabetic ulcerative wounds in Nigerian hospitals, although statistical corroboration is lacking [19,20].

The identified bacterial strains are considered the culprits of many communicable diseases. Besides, most of them are resistant to first-line antibiotics. For instance, these species were resistant to all of the tested antibiotics under the aforementioned test conditions, regardless of their different mechanisms and scope of action. Antibiotic susceptibility was determined for the isolated strains against the extracts under test, with inhibition zone results illustrated in Tables 2–5, and the MIC was noted as depicted in Fig. 2. Similarly, the retrospective findings by Trivedi and colleagues describe that more than 60%

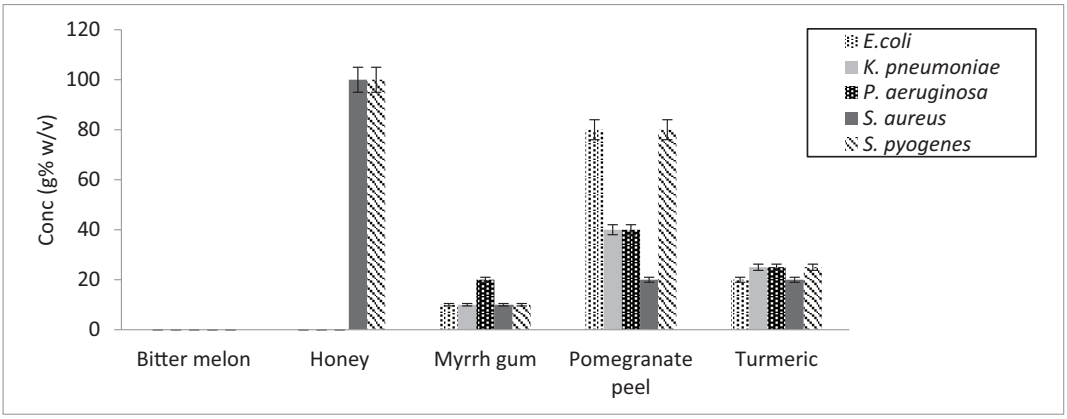
Table 1 Identification key of different bacterial isolates

Escherichia coli	Gram-negative bacilli, motile, urease negative, citrate negative, ferment lactose and form flat dry pink irregular colonies on MacConkey agar
Klebsiella pneumoniae	Gram-negative bacilli, nonmotile, urease positive, citrate positive, ferment lactose and form flat dry pink irregular colonies on MacConkey agar
Pseudomonas aeruginosa	Gram-negative bacilli, forming large, irregular and opaque bluish-green colonies on nutrient agar and colorless, nonlactose fermenting growth on MacConkey agar
Staphylococcus aureus	Gram-positive cocci, giving golden yellow colonies on mannitol salt agar
Streptococcus pyogenes	Gram-positive cocci, nonmotile, catalase negative, zone of clear β-hemolysis in blood agar

Table 2 Antimicrobial activity of bitter melon and honey against multidrug resistant bacteria

Concentration (g% w/v)	Inhibition zone diameter (mm)*				
	Escherichia coli	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Streptococcus pyogenes*
Bitter melon (100%)	0	0	0	0	0
Honey (100%)	0	0	0	17±0.61	25±1.22

Figure 2



The MIC of the tested natural products against multidrug-resistant bacteria.

Table 3 Determination of antibacterial activity of Myrrh gum against multidrug resistant bacteria

Concentration (g% w/v)	Inhibition zone diameter (mm)*				
	Escherichia coli	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Streptococcus pyogenes*
1	10±0.38	11±0.27	0.0	10±0.51	11±0.32
2	13±0.22	13±0.41	13±0.28	12±0.55	13±0.21
2.5	15±0.11	14±0.38	14±0.24	15±0.50	16±0.09

*The results are the means of three replicates±SD.

of the infections in diabetic wounds were associated with multidrug-resistant (MDR) bacteria, primarily *Pseudomonas aeruginosa* and *S. aureus* [2].

For millennia and since pre-historic times, herbs and herbal preparations have been used for wound management for disinfection, debridement, and securing a proper setting for healing [8]. Furthermore, the difficult situation that has risen with the spread of MDR bacteria led scientists to re-evaluate the measures and treatments from the preantibiotic era, especially due to their multitarget modes of action and their less liability to ensue resistant strains. By fully understanding the value of natural products and medicinal plants (i.e. mode of action, underlying pathways, and bioactive principles), different phytoconstituents made their way to be employed in standard pharmaceutical preparations, amounting to more than 60% of the different antibacterial products [10,21]. However, many factors hinder the usage of standard medicinal agents, for example, unattainability, economic burden, and cultural traditions. Thus, folkloric medicine remains the go-to remedy for a large number of people despite the high risk of market adulteration that renders the products useless to fatal [8,22].

Different tested natural extracts exhibited different *in vitro* activities against the bacterial isolates, under the described experimental conditions. Honey was active against *S. aureus* and *Streptococcus pyogenes* as illustrated in Table 2; turmeric (Table 5), myrrh gum (Table 3), and pomegranate peel (Table 4) were active against all isolates; and bitter melon showed no antibacterial activity as proved in Table 2. However, as per

Table 4 Determination of antibacterial activity of pomegranate peel against multidrug resistant bacteria

Concentration (g% w/v)	Inhibition zone diameter (mm)*				
	Escherichia coli	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Streptococcus pyogenes*
100	20.0±0.54	25.0±1.85	18.0±0.62	25.0±1.11	25.0±0.97
80	14.0±0.47	22.0±1.01	15.0±0.34	22.0±1.08	20.0±0.88
60	0.0	19.0±0.98	12.0±0.84	20.0±0.97	0.0
40	0.0	18.0±0.77	8.0±0.51	15.0±0.58	0.0
20	0.0	0.0	0.0	12.0±0.61	0.0

*The results are the means of three replicates±SD.

Table 5 Determination of antibacterial activity of turmeric against multidrug resistant bacteria

Concentration (g% w/v)	Inhibition zone diameter (mm)*				
	Escherichia coli	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Streptococcus pyogenes*
5	0.0	0.0	0.0	0.0	0.0
15	0.0	0.0	0.0	0.0	0.0
20	13±0.45	0.0	0.0	14±0.33	0.0
25	23±0.56	13±0.42	19±0.53	20±0.55	35±0.63

*The results are the means of three replicates±SD.

different records, the antimicrobial activity is considered significant if the MIC value is a maximum of 0.1 mg/ml, moderate if between 0.1 and 0.7 mg/ml, and weak if the MIC is larger than 0.7 mg/ml [23,24]. Therefore, all of the tested samples showed weak activity under the test conditions.

Per construal of the herein tested extracts, bitter melon (*M. charantia*) was the only one to show no *in vitro* antimicrobial effect on the tested MDR strains. These results are contrary to previous data that confirm the potential of the fruits and leaves against resistant Gram-positive and Gram-negative pathogens on account of the protein (e.g. α-momorcharin), polysaccharide, sesquiterpene (e.g. trans-nerolidol), and sterol content. The primary documented mechanism thereof is attributed to the ribosome-inactivating proteins [10,25]. Honey has long been described as a potent antibacterial agent upon ingestion or for wound dressing. Depending on its floral origin and how the honey is handled, its activity is the result of synergistic factors and components leading to disturbing microbial metabolic processes, for example, proliferation, quorum sensing, and different adaptive and virulence approaches. Honey is a low pH mixture of sugars, proteins (e.g. bee-defensin-1), phenols, and enzymes. In this context, the most discussed enzyme is glucose oxidase, which releases H_2O_2, which is majorly responsible for the antibacterial activity. Additionally, nonperoxide activities, connected to methylglyoxal, viscosity, osmolality, and acidity, were also discussed for their roles in the antibacterial action [26–28]. The data depicted show that honey has only been active *in vitro* against Gram-positive MDR *S. aureus* and *S. pyogenes* when used at 100% concentration, mostly owing to the water leak from bacteria in the hypertonic environment [28]. Previous observations also accord that Gram-negative organisms are more resistant to honey requiring more than 10 folds the MIC value [27], others reveal their complete resistance [26], whereas others concluded the opposite scenario with higher susceptibility of Gram-negative microbes [29]. This discrepancy further indicates the contingency between honey's biological properties and factors like the botanical source, bee health, geographical origin, and processing. Myrrh gum (*C. molmol* or *Commiphora myrrha*) comprises resin, gum (containing polysaccharides and proteins), volatile oils (containing sterols, steroids, and terpenes) in addition to bitter gourd (Momordica charantia) and other secondary metabolites (phenolic compounds and alkaloids) [30,31]. The broad-spectrum bacteriostatic and bactericidal potential has been observed and attributed to the various constituents, for example, (i) phenols attack and inactivate bacterial proteins and cell surface adhesins jeopardizing biofilm formation and quorum sensing, (ii) alkaloids block bacterial protein biosynthesis and alter the biomembrane permeability, and (iii) sesquiterpenoids were also correlated with the antimicrobial effect of myrrh [31]. The peel of pomegranate (*P. granatum* L.) is the most active antimicrobial part of the fruit on account of its tannins (e.g. ellagitannins, mainly,

α-punicalagin and β-punicalagin and ellagic acid) and flavonoids (e.g. flavonols mainly quercetin) content; hence, the extraction method and the stage of the fruit ripening play an important role [32,33]. These compounds are proven to bind to the cell wall proteins leading to cell lysis, in addition to deactivating or even denaturing other vital cell proteins with sulfhydryl functional groups. The bioactive compounds of pomegranate peel are also proven to create a nonambient environment around host cells (e.g. low pH) preventing the pathogen's attachment and compromising its virulence factors (e.g. swimming and biofilm formation) [32]. Turmeric powder, from the rhizome of *C. longa*, is one of the most popular spices worldwide and has been systematically studied since the first half of the previous century for its several versatile medicinal and nonmedicinal uses, including broad-spectrum antibacterial activity. This stems from the different active principles: alkaloids, flavonoids, curcuminoid polyphenolics (e.g. curcumin), phytosterols, saponins, tannins, and terpenoids [22,34]. Despite the poor water solubility and pharmacokinetic LADME profile, the bactericidal effect is owed to DNA replication cessation, cell membrane damage, and plasmolysis of planktonic cells in addition to quorum sensing inhibition and consequently, biofilm disruption [34,35].

Conclusion

Antimicrobial resistance is a global problem particularly facing immunocompromised patients who represent a 'high-risk population.' Nature has always been a source and inspiration for bioactive compounds of various pharmacodynamics actions, including antibacterial resources. In this report, the antibacterial activities of bitter melon, honey, myrrh gum, pomegranate peel, and turmeric are documented against several antibiotic-resistant bacterial isolates, removed from diabetic patients with foot ulcers. Indeed, the activities of these test participants were relatively weak. Yet, due to their high safety margin, they represent no risk of adverse effects, overdosing, or toxicity upon repeated application as such or after concentration to be used in nutraceuticals, antibiotic preparations, or as preservatives in the food industry.

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