Study of the antibacterial effects of Kombucha on the bacterial isolates from diabetic foot ulcer

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Abstract

Introduction: Diabetes is one of the most important metabolic diseases worldwide. Wound infections due to antibiotic resistant bacteria can cause lower limbs ulceration and amputation in diabetic patients.The present study was performed with the aim of the evaluation of antibacterial effects of cellulose disc from kombucha- on bacteria isolated from diabetic foot ulcers.

Materials and Methods: In this descriptive-analytical study,bacterial were isolated from diabetic wounds and identified based on biochemical and molecular characterization. Then the antibacterial effect of Kombucha cellulose layer wasevaluated on the isolates using disc diffusion (qualitative) and agar dilution (quantitative) methods, and the data was statisticaly analyzed.

Results: The most frequency of pathogenic bacteria that isolated in the present study from diabetic wounds were included 56% *Escherichia coli* (*E.coli*), 22% *Enterobacter cloacae* (*E. cloacae*), 6% *Citrobacter diversus* (*C. diversus*), 4% for each of *Enterobacter aerogenes* (*E. aerogenes*), *Citrobacter freundii* (*C. freundii*) and *Klebsiella pneumonia* (*K. pneumonia*), and 2% for each of methicillin-resistant *Staphylococcus aureus* (MRSA), and *Staphylococcus aureus* (*S. aureus*). TheResults of antimicrobial effect of kombucha cellulose disc showed that the disc weighing 0.5 mg was effective on all bacteria during agar disk diffusion method and the largest diameter of the growth inhibition zone was related to MRSA (27.5 mm).The minimum inhibitory concentrations (MICs) of Kombucha cellulose layer were 12.5 mg/ml on MRSA, 25 mg/ml on *S. aureus*, 75 mg/ml on *E. aerogenes*, *C. diversus* and *K. pneumonia*, 71.15 mg/ml on *E.coli*, 85 mg/ml on *E.cloacae*, and 100 mg/ml on *C. freundii*.

Conclusion: The findings of this study showed that the cellulose layer of Kombucha has excellent antibacterial effects against infectious bacteria in diabetic wounds and can be used in various medical and therapeutic targets.

Keywords: Diabetic foot ulcer, Kombucha scoby, Antibiogram, Disk diffusion method

Introduction

Diabetesis one of the metabolic disorder characterized by high blood sugar levels. Complications of this disease include cardiovascular, neurological, and kidney dysfunctions (1-2). Diabetes has adverse health consequences in human society and is a major cause of death and disability (3-4). It occurs when pancreatic langerhans islandsare unable to produce insulin or the body can not use the produced insulin effectively (1). A very common complication of diabetes is adequate wound healing or a diabetic foot

ulcer infection, which often requires long-term hospitalizations and treatment and often leads to amputation (4-5). Ischemia, neuropathy, and infection are the three most important pathologically factors which together or alone lead to complications of diabetic ulcers (6-7).

For proper treatment of diabetic foot ulcer infections, first need to know the microbiology of the infection (8-9). Infection of diabetic wounds is caused by the accumulation of pathogenic microorganisms(bacteria,fungi,viruses,andp arasites) (10). Most common pathogens interfering acute, untreated, and superficial wound infections include aerobic Gram-positive bacteria, especially *S.aureus*, and beta-haemolytic streptococci (A, B). In patients who are at risk for amputation because of chronic ulcers, infections are caused bya combination of the mentionedGram-positive bacteria along withfacultative anaerobic Gram-negative bacteria such as *E. coli*, *Proteus* and *Klebsiella* spp., and anaerobic bacteria such as *Pepto streptococcu sspp*. (11). MRSA is one of the most common bacteria that have been isolated from diabetic wounds (12). Also, the results of a study which conducted in India on patients with infectious ulcers showed that 14 out of 55 isolates of *E.coli* produced beta-lactamase (13). In another study which conducted in Spain with the aim of detection of infectious agents in diabetic foot ulcers, 102 isolated bacteria were identified, in which 68 isolates were Gram-negative bacilli (14).

foot wound infections are the most common cause of hospitalization in diabetic patients. Many causative bacteriaare resistant to a variety of antibiotics; therefore, the use of non-antibiotic treatments would be effective on preventing these antibiotic resistant infections. It is necessary to incorporate traditional medicine with modern therapeutic procedures to obtain the greatest effects inthe shortest time.Kombucha is an Asian-basedtraditional drink containing fermented black tea extract (15-17). It has traditionary been used to treat many diseases (15). A floating cellulose layer and a sour liquid environment beneath are the two major components of kombucha tea (16). Kombucha is not an individual fungus but contains a community of several yeasts and bacteria (18). The kombucha suspension is a symbiotic culture of *Acetobacter xylinum* (*A. xylinum*) and yeasts which produce a Zooglea mass. Bacteria have a unique ability to synthesize a floating cellulose network that resembles a superficial mold on a basal medium. The main material of this layer is almost pure cellulose. Thecomposition of bacterial cellulose formed by *Acetobacter* in kombucha solution is different from the composition of cellulose made by algae or plants which consists of β-D-glucan.The cellulose which secreted by *Acetobacter* contains 4-1glucopyranose bonds. The diameter of the fibrils produced is about 17 angstroms. Somestrains of *A. xylinum* also secrete xanthan, which is called Acetan (19). *A.xylinum* produces cellulose synthetase enzyme that makes low weigh cellulose microfibrils with a low bulk mass, which join together at the top surface to form a cellulose layer or disk. Kombucha microorganisms attach to the underside of this cellulose disc and create colonies. Therefore, one of the tasks of this cellulose layer is to keep the microorganisms in the vicinity of the gaseous phase of the environment and thus provide the oxygen they need. This layer enhances the ability of kombucha microorganisms to compete with other organisms for food supply. The matte color of the cellulose layer prevents the passage of ultraviolet rays, thereby protecting the underlying microorganisms from possible damage and mutations caused by the rays (20-22). Kombucha drink contains a wide range of amino acids, organic acids, enzymes,

vitamins (groups B and C) and is mineral rich.This drink is a natural source of glucuronic acid that strengthens the body by promotig oxidative metabolismandis not easily found in nature (15, 17, 20). Other benefits of kombucha are production of antibiotics and anti-cancers, as well as stimulating esophageal gastroesophageal reflux that promotes the immune system, and detoxificates and purifies blood (3). However, widespread claims about the benefits of this extract have been based on objective observations and less based on scientific evidence (23). Most researches have been conducted on kombucha supernatant and syrup-like extractand less information has been published on the antimicrobial properties of the kombucha cellulose layer. The purpose of the present study was to investigate the antibacterial activity of discs prepared from kombucha cellulose layer against infectious bacteria isolated from diabetic wounds.

Materials and Methods

Identification the Isolated Bacteria from Diabetic Wounds

In this descriptive - analytical study, 50 bacterial isolates from diabetic wounds were collected from different hospitals in Isfahan,Iran,during 3 months. The isolates were cultured on blood agar (BA) and eosin methylen blue (EMB) media (Himedia Company,India) and incubated at 35 ^{o}C for 18- 24 hours.The pure isolates were identified according to Gram staining and biochemical testing (Biometrix API KIT) (25). The final confirming identification was done by amplification of 16SrDNA gene by PCR using universal primers: DG74 (5´AGGAGGTGATCCAACCGCA3´) and RWO1 (5´AACTGGAGGAAGGTGGGGAT3´) and a RIbo-Prep PCR kit.The annealing temperature in the original PCR protocol was 55^{o}C that was modified to 50,55, and 60^{o}C in this study.The amplification reaction mixtures contained 5 µl of 10 mM Tris-HCL buffer (pH 8.3), 1.5mM $MgCl_2$, 0.001% gelatin, 1U of Taq DNA polymerase (Perkin-Elmer, Nor-walk,conn), 200µM (each) deoxynucleotid triphosphates (dATP, dCTP, dGTP and dTTP), 50 pmol of each primer, and 2 µl of the DNA that extracted from each isolate by boiling method.The PCR was carried out in a thermocycler (Amplisense Biotechnology, Russia) in30 thermal cycles consist of denaturation (94^{o}C, 1min), primer annealing (55 ^{o}C, 1 min), and extension (72 ^{o}C, 1 min); followed by a final extension (72^{o}C, 7 min). The expected 362-bpPCR products were detectedby agarose gel electrophoresis. The bands were visulizedby staining with DNA green viewer, and photographs were takenon UV light. The resulting sequences were analyzed using Chromas software version 2.1.1 and alignedwith the reported sequencesin the NCBI database (www.ncbi.nlm.nih.gov/Blast) by BLAST server (24).

Determination of Antibiotic Resistancepattern in the Isolated Bacteria from Diabetic Wounds

Antibiotic resistance pattern of the bacterial strains was evaluatedagainst the antibiotics such as cefixime(CFM,50µg), gentamicin (GM, 10 µg), ciprofloxacin (CP, 50 µg), and penicillin (P,5µg) by disk diffusion (Kerby-Bauer) method according to CLSI standard.For this purpose using sterile loops, 1 to 2 colonies of 18-24 hrs bacterial cultures were removed and added to sterile nutrient broth media to obtain 1.5×10^{8} bacterial cells per ml (equal to the turbidity of the McFarland standard 0.5 with optical density of 0.08-0.1 at the wave length of 620 nm). Then the bacteria were transferred to Müller Hinton agar (MHA, Scharlau, Spain) media by sterile loops and the antibiotic containing

standard disks were aseptically put on the mediacwith 20 mm distances from each other.Finally, the diameter of growth inhibition zones around the disks was measured after incubation for 24 hrsat 37 °C. Bacterial ATCC strains were used as positive controls. The diameters of the growth inhibitions zones were compared with standard table (PadtanTeb Company, Iran). The results were recorded in terms of sensitive, resistant or semi-sensitive for each strain (25).

Detection of Antibacterial Activity of Kombucha Cellulose Disk on the Isolated Bacteria

First the bacterial suspensions with the turbidity equal to McFarland standard 0.5 were cultivated on MHA media. After dring the media surface, the cut pieces of kombuchacellulose (approximately 6 mm diameter) were aseptically transferred to the surface of medium and incubated for 24 hrs at 37 °C. Then the diameters of growth inhibition zones around the disks were measured (25).

Detection of the Minimum Inhibitory Concentrations (MIC) by Kombucha Cellulose on the Isolated Bacteria

Agar dilution method was used. First, each 19 ml melted MHA media were distributed in sterile universal test tubes. Then 1mlof each different concentrations of kombucha cellulose (12.5, 25, 50 and 100 mg/ml) was added to each of them; completely mixed for 10 seconds and spread insterile plates. The negative control plate only contained 20 ml of MHA culture medium. The bacterial suspensions containing bacterial cells (1.5×10^4) were then inoculated in spotsonto the media and incubated for 24 hrs at 37 °C. The concentrations of kombucha cellulose that inhibited bacterial growth by more than 99% were considered as MIC (26).

Results

Biochemical and Molecular Characterization of Isolated Bacteria from Diabetic Ulcer

The amplified regions in 16S rDNA gene of the isolates formed 362 bp bands that are shown in Figure 1. The number and percentage of bacteria involved in diabetic wound infections were determined (Table1). Six Gram-negative bacteria including *E.coli*, *E.cloacae*, *E. aerogenes*, *C. diversus*, *C. freundii*, and *Klebsiella* sp. as well as one Gram-positive bacterium, MRSA, were identified. *E. coli* had the most prevalence (%56) among the diabetic wound infecting bacteria.

Table 1. The diabetic wounds infection agents and their frequency.

Isolate	Frequency	Percentage
E. coli	28	56
E. cloacae	11	22
E. aerogens	2	4
C. diversus	3	6
C. freundi	2	4
K pneumonia	2	4
MRSA	1	2
Total	50	100

Results of Antibiotic Susceptibility Against the Isolated Bacteria

The results of sensitivity and antibiotic resistance bacteria isolated from diabetic wounds using Kirby Bauer method are presented in Table (2). Most Gram-negative bacteria were sensitive to gentamicin and Cefixime, respectively

The Susceptibility of the Isolated Bacteria to Kombucha Cellulose Disk by Disk Diffusion Method

The results from measurement of growth inhibition zones are shown in table 3. The Kombucha cellulose discs were inhibited the growth of all bacterial isolates.The greatest growth inhibition zone was belonged to MRSAwith the growth inhibition zone

diameter of 27mm. *E. aerogenes*, *Kelbsiella. pneumonia*, *C. freundii*, *E. coli* and *E. Cloacae* afterward showed the highest diameter of the growth inhibition zones, respectively.

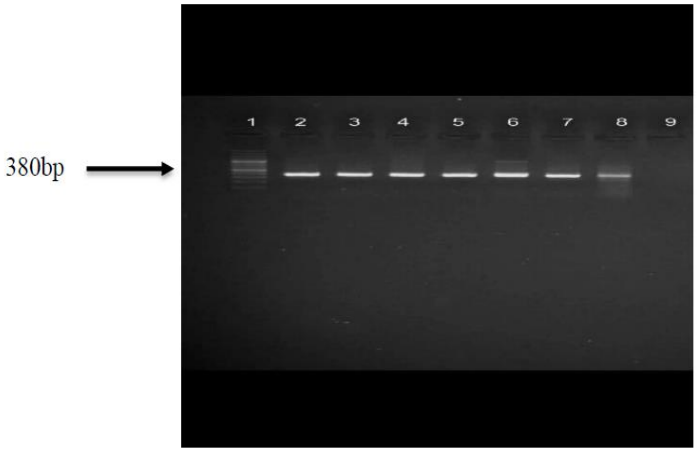

Figure 1. The Resulting PCR Product in a 1% Agarose Gel with Ethidium Bromide Staining. Lan 1: 100-bpDNA ladder, lan 2: *Enterobacter cloacea*, lan 3:*Kelebsilla pneumoniae*, 4:*Eshershia coli*, 5:*Enterobacter cloacea*,6:*Kelbsiella pneumoniae*, 7: *Citrobacter frondi*, 8:*Citrobacter diversus*, 9:*Enterobacter aeroginosa*.

Table 2. The pattern of resistance or sensitivity of bacteria against different antibiotics.

Antibiotic	P (5 µg/disk)			CP (50 µg/disk)			GM (10 µg/disk)			CFM (50 µg/disk)		
Bacteria	R*	I*	S*	R	I	S	R	I	S	R	I	S
E.coli	-	-	-	12	0	16	0	2	26	8	3	17
E.coli ATCC25922	-	-	-	0	0	1	0	0	1	0	0	1
E.Cloacae	-	-	-	0	0	11	0	0	11	0	0	11
E. aerogenes	-	-	-	1	0	1	0	0	2	1	0	1
E.aerogenes ATCC13048	-	-	-	0	0	1	0	0	1	0	0	1
C. diversus	-	-	-	0	0	3	0	0	3	2	1	0
C. freundii	-	-	-	0	0	2	0	0	2	0	2	0
C. freundii ATCC8090	-	-	-	0	0	1	0	0	1	0	0	1
Klebsiella pneumonia	-	-	-	0	0	2	0	0	2	0	1	1
K. pnemoniae ATCC13883	-	-	-	0	0	1	0	0	1	0	0	1
MRSA	0	0	1	1	0	0	0	0	1	-	-	-
S.aureus ATCC25923	0	0	1	0	0	1	0	0	1	-	-	-

*The number of resistant isolates is presented in the first row and the number of sensitive isolates is shown in the second row in front of each bacterium. Cefixime(CFM), gentamicin (GM), ciprofloxacin (CP), penicillin (P). MRSA: methicillin resistant *Staphylococcus aureus.*

The Susceptibility of the Isolated Bacteria to Kombucha Cellulose Discs by Agar Dilution Method

The results from measurement of minimum inhibitory concentrations (MICs) are shown in Table 4.

Discussion

Today, one of the most important reasons for not treated infectionsismicrobial resistance to antibiotics due to the overuse of antimicrobial drugs.

Table 3. The pattern of susceptibility of bacteria against kombucha cellulose disk by disk diffusion method.

Agent	Growth inhibition zone diameter (mm)
Escherichia coli	19.81±5.84
Escherichia coli ATCC	18.00
Enterobacter cloacae	18.58±4.17
Enterobacter aerogenes	24.75±1.12
Enterobacter aerogenes ATCC	16.16±9/00
Citrobacter diversus	20.75±1.76
Citrobacter freundii	20.70
Citrobacter ATCC	27.70
Kelbsiella pneumonia	21.00±12.2
Kelbsiella ATCC	20.00
MRSA	50.27±4.24
Staphylococcus aureus ATCC	23.60

Table 4. The pattern of minimum inhibitory concentration of kombucha cellulose discs on the bacterial isolates by agar dilution method.

Agent	Minimum inhibitory concentration (mg/ml)
Escherichia coli	71.15±25.19
Escherichia coli ATCC	50
Enterobacter cloacae	85.24±.15
Enterobacter aerogenes	75.35±35
Enterobacter aerogenes ATCC	100
Citrobacter diversus	75.35±35/00
Citrobacter freundii	100
Citrobacter ATCC	50
Kelbsiella pneumonia	75.35±35
Kelbsiella ATCC	50
MRSA	12.5
Staphylococcus aureus	25
Staphylococcus aureus ATCC	100

In other words, the use of antibiotics in high doses leads to the persistence of infection. Also, antibiotic-resistant genes transmit the resistance between generations or even from one species to another. Therefore, due to the resistance of many microbes to chemical drugs, and the severe side effects of chemical drugs attention has been paid to plant sources and the use of traditional herbs (27).

As mentioned above, kombucha has been considered in traditional medicine due to its content, including a wide range of amino acids, organic acids (acetic, lactic, glucuronic, and usnic acids), enzymes, vitamins (B and C), minerals, and antibiotics (16-17,19).

Over the past few decades, many properties of kombucha has been studied.Different efforts have been done for detection ofthe benefits of kombucha such as its antibacterial, antifungal, antiviral, anti-parasitic, and anti-cancereffectsas well as reflux improvement in esophageal gastric emptying, immune system stimulation, increasing the metabolism level, detoxification, and blood purification (17,19).The presence of acetic acid, lactic acid, gluconic acid and glucuronic acid, along with other compounds such as eosinic acid and nisin and small amounts of ethanol, gives high antimicrobial ability to Kombucha(17,19). One of the natural antibiotics in Kombucha is usnic acid.The main source of usnic acid are lichens and ithis compound has been effective against Gram-positive bacteria such as *S. aureus*, *E. faecalis*, and probably some viruses.Nisin is a bacteriocinproduced in kombucha. This compoundis mainly presents in dairy fermented products and produced by lactic

acid bacteria such as *Lactococcuslactis* subsp. Lactis (28-29). This antibiotic exerts its activity by binding to the bacterial membrane, entering the membrane layer, forming temporary pores, and interacting with lipids (29). In addition to these compounds, the antimicrobial property of kombucha can be attributed to the presence of tannins that originate from black tea (15). Esam (2014) isolated antibiotic-resistant bacteria from diabetic wounds and studied the effect of kombucha on them. Kombucha showed considerable antimicrobial activity against the isolated bacteria (25). The antibacterial effects of kombucha tea was examined on 7, 14, 21, and 28 days of incubation on the isolated bacteria. The observations showed that on the seventh day of incubation no growth inhibition zone was observed but on the 14th day the highest antimicrobial activity was seen and this effect decreased on the 21th and 28thdays (30). Dafrissens et al reported that the antimicrobial activity of kombucha against Gram-positive and Gram-negative bacteria is generally associated with acetic acid produced during fermentation (18). In the present study, for the first time, it was shown that direct use of kombucha cellulose disk had antimicrobial activity against the bacteria which isolated from diabetic woundssuch as *E. coli, E. cloacae, E. aerogenes, C. freundi, C. diversus, Klebsiella,* and *S. aureus*. Although the mechanism of activity is still unclear, it may be in partsis attributed to the beneficial characteristics of the cellulose layer produced by *A. xylinum*, one of the important bacteria exist in kombucha.The microorganisms (yeast and bacteria) in kombucha attach to the underside of the cellulose disc and microbial masses (colonies)are formed in this area (15). It can be concluded that the kombucha microbial consortium can act as a potent biofilm carrier in transmitting antibiotics and bacteriocins (by binding to cellulose disk) that play a role in the elimination of pathogens, and in addition, the solid cellulose membrane of the fungus with highly nanoporous materials, is able to pass antibiotics or other drugs into the wound and at the same time create an effective physical barrier against any external infections (20).

Conclusion

Based on the results of this study, it can be concluded that kombucha cellulose layer can show excellent antibacterial activity against bacteria that cause infections in ulcers, including diabetic ulcers, and can be used in various medical fields for wound healing.

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Conflict interests

The authors declare that they have no competing interests.

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