Study the Antimicrobial effect of Kombucha tea on bacteria isolated from Diabetic foot ulcer

دراسة التأثير المضاد للمكروبات لشاي الكمبوشا على عزلات جرثومية من قرح القدم السكري

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Abstract

The study was conducted from January to March 2012. In this study colorimetric VITEK-2 Compact system used for its accuracy and rapidity to identify isolates and to detect several antimicrobial resistances.The study also investigate the antibacterial effect of Kombucha tea on isolated bacteria from diabetic foot ulcer. The bacteria isolated were eight gram negative bacteria, namely, *Acinetobacter baumannii* 3 (2%), *Enterobacter cloacae* 5 (4%), *Escherichia coli* 13 (10%), *Klebsiella pneumoniae* 7 (6%), *Citrobacter spp.* 4 (3%), *Proteus mirabilis* 3 (2%), *Proteus vulgaris* 3 (2%) and *Pseudomonas aeruginosa* 44 (35%). Four gram positive bacterium, *Enterococcus faecalis* 6 (5%), *Staphylococcus aureus* 17 (13%), *Staphylococcus epidermidis* 13 (10%) and *Streptococcus spp*. 9 (8%). The antimicrobial activities of antibiotics showed that, all isolates are sensitive to Ciprofloxacin, Levofloxacin and Ofloxacin. The resistance to other types of antimicrobial differ with different isolate. The effect of Kombucha tea on all isolates wasclear at 7days of incubation; the diameter of inhibition was 6mm for *Acinetobacter baumannii*, *Proteus vulgaris* and *Enterococcus faecalis*. 7mm for *Enterobacter cloacae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Citrobacter spp.*, *Staphylococcus epidermidis* and *Streptococcus spp.* 8mm for *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The maximum activity of fermented tea was recorded at 14days incubation of Kombucha organism against all isolates, the diameter of inhibition was 21mm for *Acinetobacter baumannii*, 24mm for *Enterobacter cloacae*, 23mm for *Escherichia coli*, *Staphylococcus epidermidis* and *Streptococcus spp*., 16mm for *Klebsiella pneumoniae*, 22mm for *Citrobacter spp.* and *Enterococcus faecalis*, 25mm for *Proteus mirabilis* and *Staphylococcus aureus*, 20mm for *Proteus vulgaris*, 26mm for *Pseudomonas aeruginosa*. The antibacterial activity of Kombucha tea decrease with increase incubation periods28 days.

Key Words: Diabetic foot ulcer, Vitek 2 compact, Antimicrobials, Kombucha tea.

الملخص

اجريت الدراسة في الجامعة المستنصرية، المركز الوطني للسكري في الكرخ/ وزارة الصحة، دائرة صحة بغداد، المركز التخصصي لأمراض الغدد الصم والسكري في الرصافة، في مدينة بغداد، للفترة من كانون الثاني الى اذار 2012. في هذه الدراسة تم استخدام نظام VITEK-2 Compact وذلك لدقته وسرعته في تشخيص العزلات ولتقدير المقاومة للمضادات الحيوية. كما بحثت الدراسة تأثير شاي الكمبوشا على الجراثيم المعزولة من قرح القدم السكري. تم عزل ثمانية جراثيم سالبة جرام هي *Acinetobacter baumannii*3 (2%)، *Enterobacter cloacae* 5 (4%) ، *Escherichia coli* 13 (10%) ، *Klebsiella pneumoniae* 7 (6%) و*Proteus vulgaris* 3 (2%) ، *Proteus mirabilis* 3 (2%) ، *Citrobacter spp.* 4 (3%) ، Pseudomonas *aeruginosa* 44 (35%). أربع جراثيم موجبة جرام هي *Enterococcus faecalis* 6 (5%) ، *Staphylococcus aureus* 17 (13%) و *Staphylococcus epidermidis* 13 (10%) ، *Streptococcus spp*. 9 (8%). أظهرت نتائج أختبار الحساسية للمضادات الحيوية أن كل العزلات حساسة للمضادات Ciprofloxacin ، Levofloxacin و Ofloxacin.المقاومة لبقية المضادات الحيوية اختلفت باختلاف العزلات. أن تأثير شاي الكمبوشا على جميع العزلات كان واضح بعد سبعة ايام من الحضن، حيث كان قطر التثبيط 6 مليمتر لجرثومة *Acinetobacter baumannii* ، *Proteus vulgaris*و *Klebsiella* ، *Escherichia coli* ،*Enterobacter cloacae* 7مليمتر لجرثومة *Enterococcus faecalis* . 8 مليمتر لجرثومة *Streptococcus spp* و *Staphylococcus epidermidis* ، *Citrobacter spp.* ، *pneumoniae* *Staphylococcus aureus* و *Pseudomonas aeruginosa* ، *Proteus mirabilis* . أن الفعالية القصوى للشاي المخمر سجل بعد اربعة عشر يوم من حضن الكمبوشا ضد كل العزلات، حيث كان قطر التثبيط 21 مليمتر لجرثومة *Acinetobacter baumannii* ، 24 مليمتر لجرثومة *Enterobacter cloacae*،23 مليمتر لجرثومة *Escherichia coli* ، *Staphylococcus epidermidis* و *Streptococcus spp*. 16 مليمتر لجرثومة *Klebsiella pneumoniae* . 22 مليمتر لجرثومة *Citrobacter* *spp.* و *Enterococcus faecalis* . 25 مليمتر لجرثومة *Proteus mirabilis*و *Staphylococcus aureus* . 20 مليمتر لجرثومة *Proteus vulgaris*. 26 مليمتر لجرثومة *Pseudomonas aeruginosa*. أن الفعالية المضادة للجراثيم لشاي الكمبوشا تقل مع زيادة فترة الحضن (28 يوم).

الكلمات المفتاحية: قرح القدم السكري نظام Vitek 2 compact، المضاد للمكروبات، شاي الكمبوشا

Introduction

Diabetic foot ulceration and infections are a major medical, social, economic problem and a leading cause of morbidity and mortality, especially in the developing countries [1,2,3]. Fifteen percent of all diabetics develop a foot ulcer at some point in their lives which is highly susceptible to infections and that spreads rapidly, leading to overwhelming tissue destruction and subsequent amputation [4]. The major predisposing factor to foot ulceration leading to infection is usually related to peripheral neuropathy [5]. Mostly the diabetic foot infections are mixed bacterial infections [6,7] and the proper management of these infections requires appropriate antibiotic selection based on culture and antimicrobial susceptibility testing. Infection with multidrug resistant organisms may increase the duration of hospital stay, cost of management and may cause additional morbidity and mortality [8]. Early diagnosis of microbial infections is aimed to institute the appropriate antibacterial therapy to avoid further complications.

A series of the VITEK systems (BioMeriux, Marcy l'Etoile, France) has been a fully automated instrument that provides species identification (ID) andantimicrobial susceptibility testing (AST) for a variety of clinical isolates, and are presently used in many clinical microbiologylaboratories worldwide. During the past 3 decades, several revisions have been introduced to the system, resulting in a stepwise improvement of the system performance. Recently, extensive revisions, including reintroduction of colorimetric reading in lieu of fluorescence technology, and addition of several biochemical substrates and taxa covered by thebroadened database comparable with the well-established API series (BioMeriux) are created [9, 10 ,11]. The efforts have been focused upon the accurate ID, in particular, to solve its inherent weakness in the IDs of glucose-nonfermentative Gram-negative rods (GNR) and members of the family Streptococcaceae [12].

Kombucha is a symbiotic association of bacteria (*Acetobacter xylinum* and *Bacterium gluconicum*) and yeast strains (*Zygossacchromyces kombuchensis*, *Pichia fluxum* and *Saccharomyces sp*.) [13]. The variation of its composition could be due to geographic, climatic and cultural conditions as well as diversity of local species of wild yeasts and bacteria [14]. These microorganisms are able to grow in culture medium formed of tea infusions (black, mate and green), supplemented by a carbon source. The broth fermented is called "tea fungus" and is originally from the north-east of China (Manchuria). The beverage was introduced in Russia by oriental merchants and then into Eastern Europe and Europe around the turn of this century. This refreshing beverage tasting like sparkling apple cider is often produced at home by fermentation using a tea fungus passed from house to house [15]. The fermentation and oxidation processes starts, when the tea fungus is placed in a freshly prepared infusion of tea and sugar. When grown in sucrose medium, colonies of yeast break the sucrose in glucose and fructose, than produce carbon dioxide and ethanol, which are oxidized to acetaldehyde by bacteria of the colonies. The tea fungus produces many other substances, like gluconic acid and vitamins, which with the supply of tea nutrients, give the drink its unusual flavor and healing properties. The glucose is polymerized and produces cellulose and hemicelluloses [16,17]. A wide range of flavor compounds, including alcohols, aldehydes, ketnes, esters and amino acids have been identified from fermented broth [18]. The fermentation using Kombucha colonies is composed of two portions, a floating cellulose pellicle layer, formed during the fermentation by A. xylinum, and the sour liquid broth (fermented broth) [19]. The fermentation using Kombucha as a biological agent is conducted at ambient temperature for up to 7-10 days and produces a carbonated fermented broth, softly acid and with low concentration of ethanol. This broth presents beneficial effects, such as antibiotic properties, regulation of gas-gastric, intestinal and glandular activities, relief of joint rheumatism, gout and hemorrhoids, positive influence on the cholesterol level, arteriosclerosis, toxin excretion and blood cleansing, diabetes, and aging problems, and it has been claimed to be a prophylactic and therapeutic beneficial agent to human health, from weight loss to curing cancer [20,21]. The beneficial effects of Kombucha tea are attributed to the presence of tea polyphenols, gluconic acid, glucuronic acid, lactic acid, vitamins, amino acids, antibiotics and a variety of micronutrients produced during the fermentation [22].

Materials and Methods

Discharge from margins and edges of ulcer was collected with help of two sterile swabs, one for gram stain and one for culture before antiseptic dressing was applied.one hundred fiftyswabawrer collected, 50swaba from patients admitted to Al-Mustansiriya University, National Center for Diabetic (Al-Karkh).100 swabs from pacients admitted toHealth Ministry, Baghdad Health Office, Specialist Center for Endocrine glands & Diabetic Diseases (Al-Rusafa).Then swabs were immediately transported to the laboratory for culture. Samples obtained with swab sticks were streaking onto surface of nutrient agar, Blood agar and MacConkey agar media. The plates were incubated at 37°C for 24-48hrs. The identification of isolates was based on microscopic morphology, staining characteristics, culture and biochemical properties using Vitek 2 compact BioMeriux Company. [23].

Preparation of Kombucha Tea ferment (KT)

Starter culture of Kombucha (Khubdat Al-Humza) was of unknownorigin and was provided by Iraqi citizen. Kombucha was prepared by adding 100g/L (10%) weight/volume sucrose to water that had been just boiling for 15 minutes. Subsequently, black tea (Apple tea, UAE, 0.5% w/v) was added and allowed to steep for 15 minutes and then filtered through a sterile sieve. The tea was then cooled to 25°C, and 400mL of tea was aliquoted into a 750mL glass bottle that had been previously sterilized at 121°C for 20 minutes. The tea broth was inoculated with 5g of freshly grown tea fungus that had been cultured in the same medium for 14 days, and the bottle was covered with sterile tissue paper towels to allow aeration. Fermentation was carried out in a dark incubator at 25°C [16].

Antimicrobial Activity of Kombucha tea

Antimicrobial activity was demonstrated by agar diffusion assay. Mueller Hilton agar medium 20 mL was poured into each Petri dish 90 mm diameter. Suspensions 100 μL of target strain cultured for 24 h were spread on the plates uniformly, and wells of 9 mm diameter were made with a sterile cork porer. Kombucha samples were centrifuged at 40000*g* force (Du Pont centrifuge, Sorvall RC-5B) for 15 min to remove cell debris. Sterile supernatant was obtained by filtering the supernatant through a sterile microfilter (Millex-GV filter, 0.22 μm pore size, Millipore). Sterile samples 100 μL were then transferred into the wells of agar plates inoculated with target strains. The plates were then incubated at 37 °C. The diameter of the inhibition zone was measured after 12-15 h. Fermented tea sample was taking after 0, 7, 14, 21 and 28 days of incubation [16].

Results

The bacteria isolated from diabetic foot ulcer samples are shown in Table (1). eight gram negative bacteria, namely,*Acinetobacterbaumannii*3 (2%), *Enterobacter cloacae* 5 (4%), *Escherichia coli* 13 (10%),*Klebsiella pneumoniae*7 (6%),*Citrobacter spp.* 4 (3%), *Proteus mirabilis* 3 (2%), *Proteus vulgaris* 3 (2%) *and Pseudomonas aeruginosa* 44 (35%),and four gram positive bacterium, *Enterococcus faecalis*6 (5%), *Staphylococcus aureus* 17 (13%), *Staphylococcus epidermidis* 13(10%) and *Streptococcus spp.* 9 (8%) were isolated from the diabetic foot ulcer.

Table (1): Bacteria isolated from diabetic foot ulcer.

Isolate	No.	%
Acinetobacter baumannii	**3**	**2**
Enterobacter cloacae	**5**	**4**
Escherichia coli	**13**	**10**
Klebsiella pneumoniae	**7**	**6**
Citrobacter spp.	**4**	**3**
Proteus mirabilis	**3**	**2**
Proteus vulgaris	**3**	**2**
Pseudomonas aeruginosa	**44**	**35**
Enterococcus faecalis	**6**	**5**
Staphylococcus aureus	**17**	**13**
Staphylococcus epidermidis	**13**	**10**
Streptococcus spp.	**9**	**8**
Total	**127**	**100**

Table (2) shows the antimicrobial activities of commercially prepared antibiotics on the bacterial isolates. *A. baumannii* was resistant to Ampicillin, Chloramphenicol, Erythromycin, Gentamicin and Oxacillin. An *Enterobacter cloacae*was resistant to Ampicillin, Chloramphenicol, Erythromycin and

Gentamicin. *E. coli* was resistant to Chloramphenicol, Erythromycin and Gentamicin. *Klebsiella pneumoniae* and *Citrobacter spp*. were resistant to Ampicillin, Chloramphenicol, Erythromycin, Gentamicin and Oxacillin. While *Proteus mirabilis* and *Proteus vulgaris* were resistant to Ampicillin, Erythromycin and Gentamicin. *Pseudomonas aeruginosa* was resistant to Ampicillin, Erythromycin, Gentamicin and Oxacillin. *Enterococcus faecalis* was resistant to Ampicillin, Erythromycin, Gentamicin and Oxacillin. *Staphylococcus aureus* showed resistant to Ampicillin, Chloramphenicol, Erythromycin and Gentamicin. *Staphylococcus epidermidis* and *Streptococcus spp.*were resistant to Ampicillin, Chloramphenicol, Erythromycin, Gentamicin and Oxacillin.

Table(2): Susceptibility tests of antibiotics on bacteria isolated from diabetic foot ulcer.

Bacterial Isolates	Resistance in percentage							
	AMP	CHL	CIP	ERY	GEN	LEV	OFX	OXA
Acinetobacterbaumannii	54	57	40	60	51	47	36	50
Enterobacter cloacae	62	50	45	55	60	46	32	47
Escherichia coli	46	62	33	60	65	43	21	42
Klebsiellapneumoniae	58	50	29	64	58	42	20	51
Citrobacter spp.	61	50	29	70	51	35	26	50
Proteus mirabilis	76	33	25	57	52	45	34	46
Proteus vulgaris	50	43	39	55	51	40	32	44
Pseudomonas aeruginosa	63	40	43	60	63	47	26	50
Enterococcus faecalis	67	47	42	77	67	49	29	50
Staphylococcus aureus	72	51	32	59	70	29	26	37
Staphylococcus epidermidis	68	53	41	65	77	41	32	55
Streptococcus spp.	70	50	39	66	80	33	21	50

AMP- Ampicillin, CHL- Chloramphenicol, CIP- Ciprofloxacin, ERY- Erythromycin, GEN- Gentamicin, LEV- Levofloxacin, OFX- Ofloxacin, OXA- Oxacillin.

Table (3) shows that Kombucha tea has effective antibacterial activities on the diabetic foot ulcer isolates as indicated by the diameter of their zone of inhibition. The effect of Kombucha tea on all isolates was at 7days of incubation, the diameter of inhibition was 6mm for *Acinetobacter baumannii*, *Proteus vulgaris* and *Enterococcus faecalis*. 7mm for *Enterobacter cloacae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Citrobacter spp.*, *Staphylococcus epidermidis* and *Streptococcus spp.* 8mm for *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The maximum activity of fermented tea was recorded at 14days incubation of Kombucha organism against all isolates, the diameter of inhibition was 21mm for *Acinetobacter baumannii*, 24mm for *Enterobacter cloacae*, 23mm for *Escherichia coli*, *Staphylococcus epidermidis* and *Streptococcus spp*., 16mm for *Klebsiella pneumoniae*, 22mm for *Citrobacter spp.* and *Enterococcus faecalis*, 25mm for *Proteus mirabilis* and *Staphylococcus aureus*, 20mm for *Proteus vulgaris*, 26mm for *Pseudomonas aeruginosa*. The antibacterial activity of Kombucha tea decrease with increase incubation periods (28 days).

Table (3): Antimicrobial activities of Kombucha tea on bacteria isolated from diabetic foot ulcer.

Bacterial isolates	Incubation Periods of Kombucha colonies				
	0day I.Z(mm)	7days I.Z.(mm)	14days I.Z.(mm)	21days I.Z.(mm)	28days I.Z.(mm)
Acinetobacter baumannii	0.0	6.0	21.0	19.0	10.0
Enterobacter cloacae	0.0	7.0	24.0	20.0	11.0
Escherichia coli	0.0	7.0	23.0	20.0	11.0
Klebsiella pneumoniae	0.0	7.0	16.0	20.0	11.0
Citrobacter spp.	0.0	7.0	22.0	18.0	10.0
Proteus mirabilis	0.0	8.0	25.0	22.0	13.0
Proteus vulgaris	0.0	6.0	20.0	13.0	9.0
Pseudomonas aeruginosa	0.0	8.0	26.0	22.0	13.0
Enterococcus faecalis	0.0	6.0	22.0	19.0	10.0
Staphylococcus aureus	0.0	8.0	25.0	22.0	13.0
Staphylococcus epidermidis	0.0	7.0	23.0	20.0	12.0
Streptococcus spp.	0.0	7.0	23.0	20.0	12.0

I.Z. = Inhibition Zone.

Discussion

Acinetobacter baumannii, *Enterobacter cloacae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Citrobacter spp.*, *Proteus mirabilis*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*,

Staphylococcus epidermidis and *Streptococcus spp.* were isolated from patients with diabetic foot ulcer. Similar organisms have been reported [24,25].

Overall, the evaluation results of the newly redesigned colorimetric VITEK-2 ID impressed us by the performance because more than 98% of the isolates were correctly identified to the species level without any further additional tests. Also, our results indicated that the current VITEK-2 has overcome its inherent weakness in IDs of streptococci and glucose-no fermentative GNR. Until present, API test strips has been long considered as the be gold standard in ID test [3,4]. But the accuracy of the VITEK-2 was finally estimated to be 98.3%, compared with 97.5% by the respective API test strips. Our results were highly consistent with a series of evaluation results recently published for GPC [9], GNR [12].

The antimicrobial activities of commercially prepared antibiotics on the bacterial isolates showed, the all isolates are sensitive to quinolones (Ciprofloxacin, Levofloxacin and Ofloxacin) this agree with many references which showed the almost bacteria isolated from diabetic foot ulcer are sensitive to quinolones compounds [26,27]. The resistant to other types of antimicrobial differ with different isolate, these agree with [5]. The interpretation of results due tofew use of Quinolones in Baghdad hospitals compared with other antimicrobials such as Ampicillin, Chloramphenicol, Erythromycin, Gentamicin and Oxacillin.

The results of this study shows that Kombucha tea has antimicrobial actions on all bacteria isolated from diabetic foot ulcer. Table (3) shows that all the isolates were sensitive to Kombucha tea even at 7 days of incubation. The interpretation of results due to decreased the pH of the Kombucha tea with fermentation time. During the fermentation process, yeasts and bacteria metabolize sucrose into a number of organic acids, such as acetic acid and gluconic acid. Due to an increased concentration of these organic acids, the pH decreased from 5 to 2.5 within 6 days of fermentation and remained stable thereafter. These observations are in agreement with the findings of other studies [13,16]. The inhibition of bacterial growth caused by acid shock, low pH.

The maximum antimicrobial effect of Kombucha tea noted at 14 days of incubation table 3, in this period the biggest inhibition zones were recorded, this agreement with several references [14,15,16] which found a slight secondary growth of bacteria found in Kombucha tea was observed after 12 days of fermentation, likely due to multiplication of acid-tolerant bacterial strains therefore the produce of inhibine increases in this period. The antibacterial activity of Kombucha tea decrease with increase incubation periods (28 days) this agreement with several references [17,18] in which interpretation the results due to littleness Carbone source and other nutrients required for Kombucha growth.

The antimicrobial activity of Kombucha under different incubation periods studied against a number of pathogenic microorganisms which causes diabetic foot ulcer, Kombucha had its strongest antimicrobial effects,and this implies the existence of an antimicrobial component other than acetic acid and large proteins. There are numerous reports that the polyphenols/ tannins extracted from tea inhibit a broad spectrum of Gram-positive and Gram-negative bacteria. Among the catechins tested, epigallocatechin, epicatechingallate, and epigallocatechingallate have been found to be inhibitory for the growth of *S. aureus* and *V. cholerae* [16]. Other studies [17,18,20] reported that the extracts of green and black tea can inhibit *Cm.jejuni*, *E. coli*, and *H. pylori*. Recently, [19] have tested the antimicrobial activity of Kombucha as well as normal tea extracts prepared at different concentrations and found that the inhibitory effects of Kombucha increased with the tea concentration. In our studies, the concentration of tea broth was 0.5% for the preparation of Kombucha. The polyphenol/ tannin level in such a low concentration of tea was unlikely to have an inhibitory effect against the target microorganisms.Hence, these findings suggest the presence of an antimicrobial compound other than acetic acid, large proteins, and catechins in Kombucha. Antimicrobial activity increased with fermentation time until 21days [21]. As seen in almost all cases tested. This also implies that the active antimicrobial components are very likely metabolites produced by the bacteria and/or yeasts responsible for the fermentation of Kombucha .At present a characterization of antimicrobial compounds is in progress [22].

The present study concluded that, Kombucha tea is the best alternative medicine material has very good antimicrobial activity against pathogenic bacteria. We recommend further studies on the possibility on using Kombucha tea as bio agent in treatment diabetic foot ulcer. This agent has three important characteristics, antimicrobial activity, reducing glucose levels in blood [28] and used for medical

purposes in skin therapy. The cellulosic pellicle formed mainly by *Acetobacterxylinum* during the fermentation of tea has been used as a temporary skin substitute on burns and in other skin injuries [29].

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