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Moran GJ, Amii RN, Abrahamian FM, Talan DA (2005). Methicillinresistant Staphylococcus aureus in community-acquired skin infections. Emerg. Infect. Dis. 11: 928-930.

Pitout JDD, Church DL, Gregson DB, Chow BL, McCracken M, Mulvey M, Laupland KB (2007). Molecular epidemiology of CTXM-producing Escherichia coli in the Calgary Health Region: emergence of CTX-M-15-producing isolates. Antimicrob. Agents Chemother. 51: 1281-1286.

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Full Length Research Paper

Purification and characterization of a bacteriocin produced by *Lactococcus lactis* subsp. *lactis* PD6.9

Margarete Alice Fontes Saraiva[1]*, Ingolf Figved Nes[3], Maria Cristina Baracat-Pereira[2], Marisa Vieira de Queiroz[1], Hilário Cuquetto Mantovani[1] and Célia Alencar de Moraes[1]

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In this study, a bacteriocin produced by *Lactococcus lactis* subsp. *lactis* PD6.9 was purified, characterized and identified. The bacteriocin was purified to homogeneity from culture supernatant by cation exchange and reversed-phase liquid chromatography, and its molecular weight was determined by mass spectrometry. The presence of the nisin gene was confirmed by polymerase chain reaction (PCR) and DNA sequencing. The gene showed that it was a natural nisin variant, nisin Z, as indicated by substitution of an asparagine residue for histidine at position 27. The purified bacteriocin was biochemically pure, and the molecular weight was approximately 3329.571 Da. The peak of nisin Z production by *L. lactis* PD6.9 occurred after 5 h of culture during stationary phase. This bacteriocin demonstrated inhibitory activity towards significant foodborne pathogens and *Staphylococcus aureus* strains isolated from dairy cattle diagnosed with mastitis, it may be useful for future applications.

Key words: Antimicrobials, *Lactococcus lactis*, bacteriocins, identification, inhibitory activity.

INTRODUCTION

Lantibiotics are antimicrobial peptides that have attracted widespread scientific attention as promising safe and natural food additives and as potential therapeutic agents to combat medically significant bacteria and their multi-drug resistance (Field et al., 2008). These ribosomally synthesized peptides are distinguished by the presence of post-translationally modified amino acids such as dehydroalanine (Dha), dehydrobutyrine (Dhb) and eponymous lanthionine (Lan) and *β*-methyllanthionine (MeLan) formed by thioether linkages between dehydrated amino acid residues and neighboring cysteines (Rink et al., 2007). Nisin A is a lantibiotic produced by *Lactococcus lactis*, has already been employed as a food preservative for long time and is licensed by 48 countries around the world (Delves-Broughton, 1990) and is one of the few bacteriocins to have been applied commercially (Bierbaum and Sahl, 2009). This peptide is suggested to be effective

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against many Gram-positive bacteria, including food-borne pathogens such as staphylococci, bacilli, clostridia and mycobacteria (Field et al., 2010). Some natural variants of nisin have been described as nisin Z (Mulders et al., 1991; De Vos et al., 1993), nisin F (De Kwaadsteniet et al., 2008), nisin Q (Zendo et al., 2003) produced by strains of *L. lactis,* nisin U (Wirawan et al., 2006) and nisin U2 (Piper et al., 2010) produced by *Streptococcus uberis* and *Streptococcus agalactiae*, respectively.

Potentially, the most significant application of lantibiotics may be in the treatment of antibiotic resistant pathogens. Nisin A has been shown to be active against a number of multidrug-resistant Gram-positive pathogens (Goldstein et al., 1998; Severina et al., 1998), including a wide range of mastitis-causing pathogens (Cotter et al., 2005a).

Mastitis is the inflammation of the mammary gland in response to bacterial invasion. Clinical mastitis results in alterations in milk composition and appearance and decreased milk production (Wu et al., 2007). Because of increased antibiotic resistance of mastitis pathogens (Wang et al., 2006), reduced responses to antibiotic therapy have become very common in veterinary practice (Cao et al., 2007). In addition, loss of milk due to discarding milk contaminated with antibiotics has been the reason why treatment of mastitis is not suggested during lactation (Wu et al., 2007).

We have previously described a natural isolate of *L. lactis* subsp. *lactis* strain PD6.9, which produces a bacteriocin possessing some important features (De Carvalho et al., 2006). Here, we reported the purification and identification of this bacteriocin designated as nisin Z. Purified nisin Z was tested against foodborne pathogens and others pathogenic bacteria, including *Staphylococcus aureus* strains responsible for bovine mastitis.

MATERIALS AND METHODS

Bacterial strains and culture conditions

L. lactis subsp. *lactis* PD6.9 was cultured in M17 broth (Oxoid) supplemented with 0.4% (w v^{-1}) glucose at 30°C. All target strains were grown in brain heart infusion (BHI) broth (Oxoid) at 37°C for 12 h before tests.

Bacteriocin production

L. lactis subsp. *lactis* PD6.9 (1% inoculum, v/v, standardized to OD_{600nm} = 0.6) was grown in M17 broth with glucose and incubated at 30°C, without agitation, for 24 h. Culture samples were collected each hour and bacterial growth (absorbance values, OD_{600nm}) and changes in culture pH were determined. Preparations of the cell-free culture supernatant collected (boiled and neutralized) were serially diluted and tested against indicator *L. lactis* IL1403 for determination of bacteriocin activity (expressed as BU mL^{-1}).

Bacteriocin activity testing

Quantitative determination of the antimicrobial activity of the bacteriocin was performed using a microtiter assay method (Holo et al., 1991). A twofold serial dilution (in medium) with 100 µL bacteriocin samples were prepared in a microtiter plate well containing 50 µL of culture medium to which 150 µL of a diluted culture of the target bacteria (approximately 10^6 viable cells mL^{-1}) were added. The plate was incubated for 12 h, after which growth inhibition was measured turbidometrically at 620 nm with a microtiter plate reader (Labsystems iEMS reader MF; Labsystems, Helsinki, Finland). One bacteriocin unit (BU) was defined as the amount of bacteriocin that inhibited 50% growth of the target microorganism under these conditions.

To study the antimicrobial spectrum of the bacteriocin produced by *L. lactis* subsp. *lactis* PD6.9, a wide range of target organisms (Table 1) were used in the microtiter assay system.

DNA isolation, PCR and sequencing

Genomic DNA was isolated with Wizard Genomic DNA purification Kit (Promega, USA), applying the protocol for Gram-positive bacteria and using mutanolysin and lysozyme (Sigma-Aldrich, USA). Nucleotide sequencing was performed with the PCR products obtained from amplifications of genomic DNA of *L. lactis* subsp. *lactis* PD 6.9 using the following primers specific to nisin structural gene: nqf (5'-GTTCGAAGGAACTACAAAATAAATT-3') and naqzr (5'-ACAGACCAGCATTATATTTCTGC-3'); and to the *nisA* promoter region: pnisAf (5'-TTGAGTCTTAGACATACTTGAATGACC-3') and pnisAr (5'-CAATGACAAGTTGCTGTTTTCA-3'). Each PCR procedure was performed separately and differently. The PCR thermal cycle program included a pre-denaturation at 94°C for 2 min followed by 35 cycles, with a denaturation step at 94°C for 1 min, an annealing step for 30 s at 40°C (for primers sets nqf/naqzr) and 48°C (for primers sets pnisAf/pnisAr) followed by an extension step for 1 min at 72°C. A final extension was performed at 72°C for 7 min. PCR products were purified with the Gel Extraction Kit (Nucleospin® Gel and PCR clean up, Machery-Nagel, Germany) and sequenced using the BigDye Terminator v3.1 cycle Sequencing Kit and ABI Prism 377 DNA sequencing system (Applied Biosystems, United States). Sequences were aligned using BLAST software provided online by National Center for Biotechnology Information (USA).

Purification of bacteriocin

The supernatant from a 200 mL overnight culture (at 30°C in M17 supplemented with 0.4% glucose) of *L. lactis* subsp. *lactis* PD6.9 was collected. Ammonium sulfate (40 g per 100 mL) was added to the supernatant and agitated for 30 min at 4°C. The bacteriocin was then precipitated from the supernatant by centrifugation (10,000 ×*g* for 30 min at 4°C) and dissolved in 20 mL sterile distilled water, and the pH was adjusted to 3.5 with 1 M HCl. It was then passed through a 5 mL SP Sepharose Fast Flow column (GE Healthcare Biosciences, Uppsala) equilibrated with 10 mM acetic acid. The column was eluted with a stepwise gradient consisting of 10 mL each of 0.1, 0.3 and 1.0 M NaCl at 1 mL/min flow rate and stored on ice. The fractions displaying the highest bacteriocin activity were used for further purification. The purification was followed by reversed-phase chromatography using the Äkta Purifier fast protein liquid chromatography system. The most active fractions following cation exchange chromatography were applied to a reversed-phase column (Resource 15 RPC 3 mL; Pharmacia Biotechnology) equilibrated with 0.1% trifluoroacetic acid (TFA) in water. Elution was performed with a linear water-isopropanol gradient from 0 to 100% isopropanol containing 0.1% TFA (w v^{-1}). The most active

Table 1. Inhibition spectrum of nisin Z produced by *L. lactis* subsp. *lactis* PD6.9.

Indicator	Strain	Source or reference	BU (mL^{-1})
Bacillus subtilis	DSMZ347	LMGT	640
Enterococcus faecalis	v583	LMGT	160
Lactococcus lactis	IL1403	LMGT	10200
Listeria innocua	BL86/26B	LMGT	1280
Listeria innocua		CERELA	1280
Listeria ivanovvi		CERELA	640
Listeria monocytogenes		CERELA	1280
Listeria monocytogenes	Scott A	CERELA	2560
Micrococcus luteus	4698	ATCC	640
Micrococcus luteus	10240	ATCC	5120
Streptococcus pneumoniae	TIGR4	LMGT	1280
Staphylococcus aureus	ME8245-3	LMGT	2560
Staphylococcus aureus	8452	LMGT	1280
Staphylococcus aureus	4759	Pinto, 2008	2560
Staphylococcus aureus	4052	Pinto, 2008	2560
Staphylococcus aureus	4784	Pinto, 2008	1280
Staphylococcus aureus	3870	Pinto, 2008	640
Staphylococcus aureus	4119	Pinto, 2008	5120
Staphylococcus aureus	3212	Pinto, 2008	640
Staphylococcus aureus	3702	Pinto, 2008	1280
Staphylococcus aureus	4716	Pinto, 2008	2560
Staphylococcus aureus	3975	Pinto, 2008	10240
Staphylococcus aureus	3129	Pinto, 2008	1280
Escherichia coli	14763	ATCC	NI
Pseudomonas aeruginosa		LMGT	NI

ATCC, American Type Culture Collection; CERELA, Reference Center for Lactobacilli; LMGT, Laboratory of Microbial Gene Technology (UMB); NI, no inhibition.

fractions were stored at 4°C for further analysis. The susceptible strain *L. lactis* IL1403 was used as the indicator strain in biological assay for bacteriocin quantification.

Mass spectrometry

The molecular weight of the purified bacteriocin was determined by mass spectrometry. Bacteriocin samples (active fractions) were mixed 1:1 with a solution of 15 mg α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 49.9% ethanol and 0.1% TFA and deposited on a ground steel matrix-assisted laser desorption ionization target. Mass spectra were recorded in the positive reflector mode with an Ultra Flex TOF/TOF (Bruker Daltonic GmBH, Bremen, Germany), using a pulsed ion extraction setting of 40 ns and an acceleration voltage of 25 kV.

Effect of heat and proteolytic enzymes on the stability of nisin Z

Samples of fractions purified were dispensed in micro tubes and treated separately in a water bath at 100°C for 15 min and 100°C for 30 min. After, samples were cooled and the residual activity was determined. The protein nature of the antimicrobial compounds was verified by treatment with the enzymes trypsin (Sigma-Aldrich) and proteinase K (Finnzymes) at 10 mg mL^{-1} concentration, in 0.01 M phosphate buffer at pH 7.0 and were added to 0.1 mL of purified fraction sample to give a 1 mg mL^{-1} enzyme final concentration. The samples were filtered through 0.22 µm pore-size filters (millipore) and incubated for 5 h at 37°C. The reactions were stopped by boiling the mixture for 3 min. The residual activity was tested.

RESULTS

Bacteriocin production

The kinetics of microbial growth and bacteriocin production of *L. lactis* subsp. *lactis* PD6.9 are presented in Figure 1. Exponential growth of *L. lactis* subsp. *lactis* took place during a period of approximately 4 h, and bacteriocin production began during the exponential phase (Figure 1). Its activity reached a maximum level (22.000 BU mL^{-1}) at 5 h of culture during stationary phase when the pH of the medium was below 4.5 (Figure 1). After 7 h of incubation, bacteriocin titres decrease by approximately 37%, and remained constant to 24 h (Figure 1).

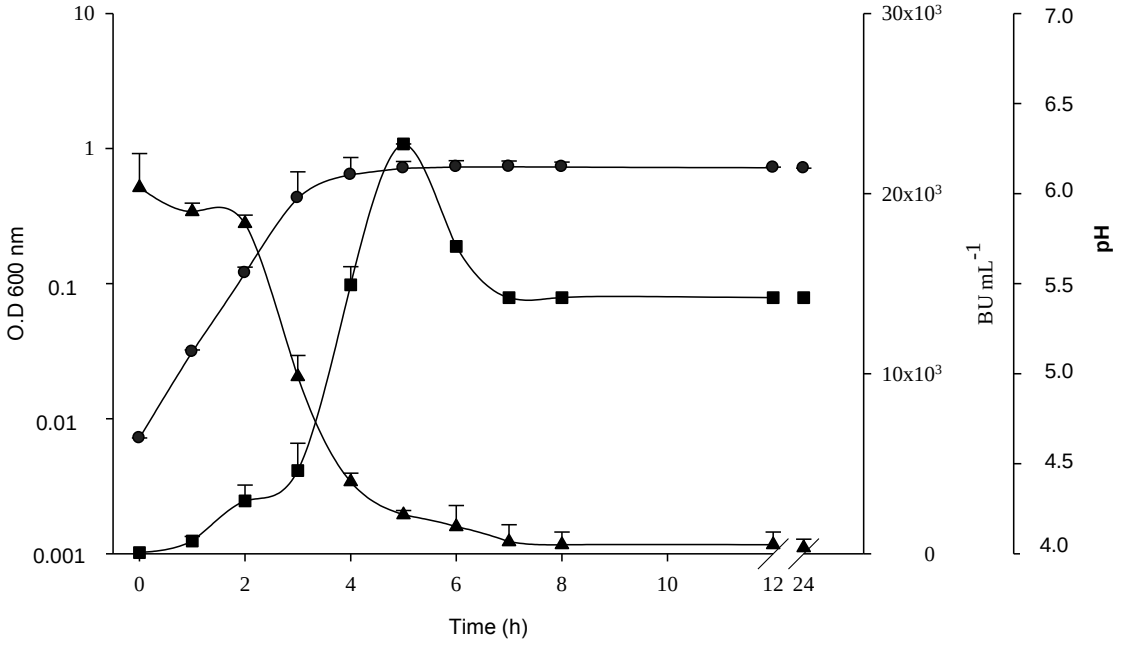

Figure 1. Production of bacteriocin during growth of *L. lactis* subsp. *lactis* PD6.9 in M17 broth at 30°C. Antimicrobial activity is presented as BU mL^{-1}(■), change in optical density (●) and pH (▲) are indicated.

Table 2. purification of the bacteriocin produced by *L. lactis* subsp. *lactis* PD6.9.

Purification step	Volume (mL)	Recovery (%)	Protein concentration (mg mL^{-1}) [a]	Antimicrobial activity (BU mL^{-1})	Specific activity (BU mg^{-1})	Increase in specific activity (fold)
Cell free culture supernatant	200	100	24.87	1.28×10^3	51.46	1.0
Ammonium sulphate precipitate	20	20	4.44	2.56×10^3	576.58	11
Ion-exchange chromatography	10	20	0.32	5.12×10^3	1.6×10^4	311
Reversed- phase chromatography	2	8	0.08	1.02×10^4	1.28×10^5	2490

[a]The protein concentration was determined by measuring the optical density at 280 nm.

Purification of bacteriocin and mass spectrometry

The cell free culture supernatant from a 0.2-L culture of *L. lactis* subsp. *lactis* PD6.9 grown overnight in M17 broth was used for bacteriocin purification. This supernatant was precipitate with ammonium sulfate and subsequently purified by cation exchange and reversed-phase chromatography (Table 2). The cell free culture supernatant contained 1280 bacteriocin units mL^{-1} as determined with the indicator strain *L. lactis* IL1403 (Table 2). The specific activity of the bacteriocin was concentrated 10-fold from the cell free culture supernatant by ammonium sulphate precipitation. This concentration step resulted in a recovery of 20% of activity. Upon a subsequent pre-purification step by ion exchange chromatography, the specific activity was about 300-fold higher than that of the cell free culture supernatant and the recovery was about 20%. The specific activity of the final purified bacteriocin eluted from the reversed-phase chromatography was about 2500-fold higher than that of the cell free culture supernatant, with a recovery of about 8%. The results of the purification procedure are summarized in Table 2.

The molecular mass of purified fractions 14 and 15 (Figure 2A) was determined by mass spectrometry to be 3329.571 Da (M+1, 3330. 6) (Figure 2B), which is close to the molecular mass of lantibiotic nisin Z, whose monoisotopic molecular mass is 3330.93 Da (Piper et al.,

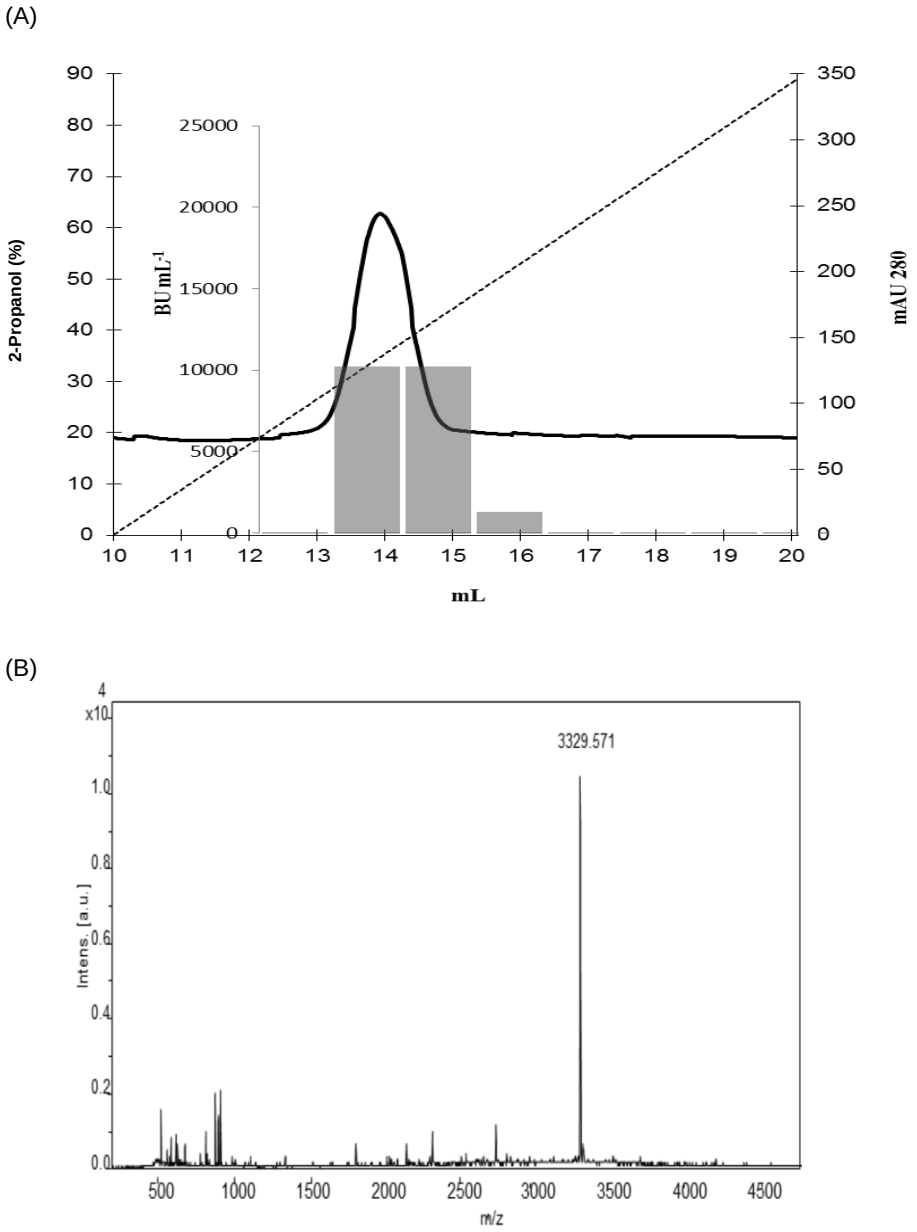

Figure 2. (A) Results of second reversed-phase chromatography of bacteriocin produced by *L. lactis* subsp. *lactis* PD6.9. Elution was performed by using a linear gradient of 0 to 100% 2-propanol containing 0.1% TFA. Solid line, absorbance at 280 nm; dashed line, isopropanol gradient; bars, bacteriocin units (BU) in active eluted fractions. (B) Mass spectrometry analysis of purified bacteriocin.

2010).

Effect of heat and proteolytic enzymes on the stability of nisin Z

The inhibitory action of purified fraction of nisin Z was inactivated when it was treated with trypsin and proteinase K. Furthermore, the activity of nisin was maintained after heat treatment at 100°C for 15 and 30 min (date not shown). These results demonstrated that nisin Z produced by *L. lactis* PD6.9 was heat stable.

PCR and sequencing nisin genes

PCR products obtained from amplifications of *L. lactis* subsp. *lactis* PD6.9 genomic DNA with primers specific to the nisin structural gene were subjected to nucleotide sequencing (Figure 3A). The results indicated that the

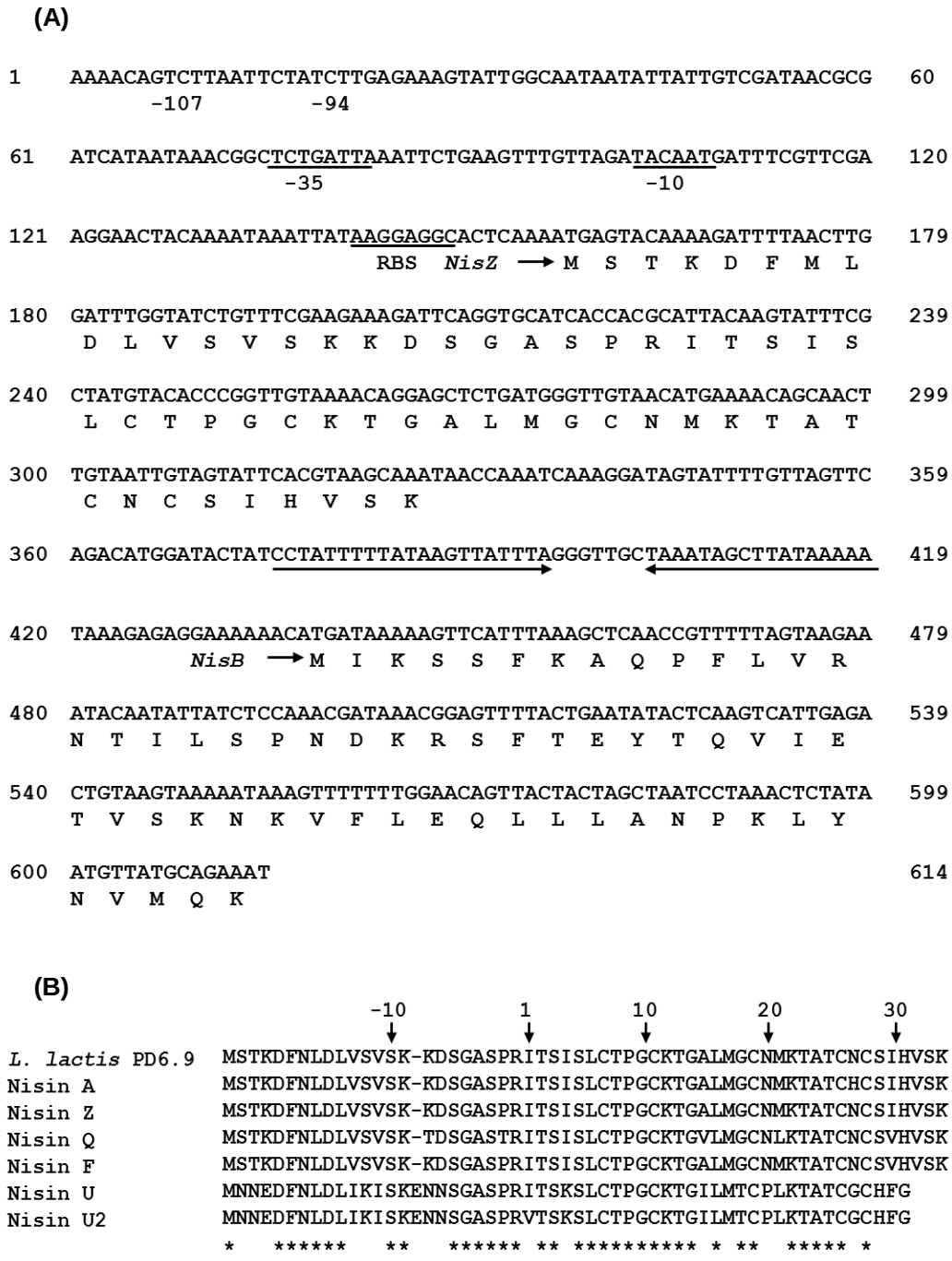

Figure 3. (A) Nucleotide sequence of the region encoding nisin Z in *L. lactis* subsp. *lactis* PD6.9 and deduced amino acid sequence. The putative -35 and -10 promoter regions and a putative ribosome binding site (RBS) are underlined. (B) Alignment of nisin Z in *L. lactis* subsp. *lactis* PD6.9 and homologous sequences of nisin A, Z, Q, F, U and U2 are obtained from GenBank data. Identical amino acid residues are indicated with an asterisk.

sequence of the PD6.9 nisin gene was identical to that of nisin Z (GenBank accession number AB375441.1). Homology with nisin A (GenBank accession number HM219853.1) was also recorded, except for a C-to-A transversion at position 148 (Figure 3A). This resulted in an asparagine (AAT) residue at position 27 in the nisin peptide instead of histidine (CAT). The deduced amino acid sequence showed complete similarity (100% identity) to nisin Z (GenBank accession number P29559.1). This indicates that the bacteriocin produced by *L. lactis* subsp. *lactis* PD6.9 is a natural variant, nisin Z, as shown in Figure 3B. The nucleotide sequence of

the PCR fragment (amplified with primers pnisAf and pnisAr) containing the *nisA* promoter region had 99% identity to the sequence recorded for the promoter region encoding nisin Z (Gen Bank accession number Y13384.1). It has a consensus promoter characterized by sequences at -35 and -10 that are spaced by an average of 17 nucleotides. The promoter region upstream of the structural *nis* gene contains a TCT direct repeat with an 8-bp spacer region at positions -39 to -26 upstream of the transcription start site. It also contains a second TCT-N_8-TCT motif present upstream of the structural *nisZ* gene at positions -107 to -94 (Figure 3A).

Assay of bacteriocin activity

The inhibitory spectrum of nisin produced by *L. lactis* subsp. *lactis* PD6.9 is presented in Table 1. Members of several species of Gram-positive bacteria (*Lactococcus*, *Bacillus*, *Enterococcus*, *Listeria*, *Micrococcus*, *Streptococcus*, *Staphylococcus*) were susceptible to nisin Z, but species Gram-negative (*Escherichia coli* ATCC 14763 and *Pseudomonas aeruginosa*) were not affected (Table 1). The bacteriocin unit concentration varied considerably among the different target strains, with *L. lactis* IL1403, *Micrococcus luteus* ATCC 10240, *S. aureus* 4119 and *S.s aureus* 3975 seeming to be the organisms most sensitive to nisin Z, whereas *Enterococcus faecalis* v583 appeared to be less sensitive (Table 1). All the *S. aureus* strains (4749, 4052, 4784, 3870, 4119, 3212, 3702, 4716, 3975 and 3129) isolated from dairy cattle diagnosed with mastitis were sensitive to nisin Z (Table 1).

DISCUSSION

In the last few years, a variety of bacteria such as lactic acid bacteria have attracted attention for their production of compounds with potential uses in many fields. In this investigation, we have carried out the identification and purification of the antimicrobial compound produced by a naturally fermented salami isolate of lactic acid bacteria, *L. lactis* subsp. *lactis* PD6.9 (Maciel, 1998). In fact, the nisin production phenotype has been widely found among *L. lactis* strains from cheese, raw milk, grain, fish, fermented vegetable and river (Choi et al., 2000; Zendo et al., 2003; Mitra et al., 2005; De Kwaadsteniet et al., 2008). However, *L. lactis* PD6.9 was isolated from fermented salami, indicating that this product could be a good source of strains displaying enhanced outputs.

The bacteriocin produced by *L. lactis* PD6.9 displayed secondary metabolite kinetics, because the bacteriocin was produced during exponential growth phase and reached a maximum level at stationary phase. Extending stationary phase resulted in a decrease in bacteriocin production. This decrease could be due to the activity of extracellular endogenous proteases induced within this growth phase.

Development of three steps purification procedure allowed the separation of bacteriocin and the reliability of each step were demonstrated by significant increase in the specific activity of bacteriocin. After mass spectrometry analysis of the purified bacteriocin, the apparent molecular mass was confirmed to be 3329.571 Da (M+1, 3330.6), corresponding the native form of nisin Z.

Heat stability and protease sensitivity is a key criterion for the characterization of an inhibitory substance such as bacteriocin. The heat stability and no activity of nisin Z was produced by *L. lactis* PD.69 when treated with trypsin and proteinase K. These were characteristics similar to those of other nisins (Matsusaki et al., 1998; Mitra et al., 2005; De Kwaadsteniet et al., 2008).

The deduced amino acid sequence of the PD6.9 nisin showed that it contained an asparagine at position 27 instead of a histidine as in nisin A. A BLAST search of GenBank sequences indicated that the PD6.9 nisin is a variant nisin Z. De Vos et al. (1993) reported that the His27Asn substitution resulted in a higher diffusion rate for nisin Z, that may be of practical significance, since many products to which nisin A is applied are diffusion limited.

Natural variants of a number of lantibiotics have been described (Cotter et al., 2005a). The existence of natural variants suggests that the identity of amino acids present at certain locations is flexible and it thus may be possible to generate mutants. These natural variants may highlight regions of lantibiotics that demonstrate a greater propensity and permissiveness to change, while comparisons of more distantly related peptides permit the identification of conserved regions that are likely to be essential for activity (Cotter et al., 2005a). In addition, nisin variants may have potential as novel antibiotics because, generally, it is not recommended to use the same compound both for food preservation and for antibiotic treatment (Lubelski et al., 2008).

The promoter sequences of *nisZ* of the PD6.9 strain was identical to be nisin A (GenBank accession number HM219853.1), contain a partially conserved region which could be involved in the transcriptional control function, the TCT-N_8-TCT motif present upstream of structural gene *nisZ* (Figure 2A). Chandrapati and O'Sullivan (2002) reported that the TCT-N_8-TCT motif present upstream of the structural *nisZ* gene is supposed to be involved in a co-operative binding of the NisR response regulator of the NisRK two-component regulatory system involved in the transcriptional control function (Figure 3A). They also reported a second TCT-N8-TCT motif present upstream of *nisA* at positions -107 to -94, which was also shown in our results. This TCT repeat, together with the first one, is involved in the optimal binding of NisR (Trmčić et al., 2011).

The spectrum of target bacteria against which the nisin Z produced by PD6.9 strain is effective is also interesting.

Among the bacteria tested, the pathogens *L. monocytogenes*, *S. pneumoniae* and *S. aureus* were sensitive. Studies with nisin variants against a series of clinically significant pathogens have established differences in specific activities against selected targets (Piper et al., 2010). Piper et al. (2010) stated that nisin Z had an inhibitory effect against methicillin-resistant *S. aureus* (MRSA) and (heterogeneous) vancomycin intermediate *S. aureus* [(h)VISA]. In addition, most of the *S. aureus* strains that were more sensitive to nisin Z were isolated from bovine mastitis. This is significantly due to the severity of the clinical symptoms and mortality associated with this infection (Espeche et al., 2009). Mastitis is the main disease affecting dairy cattle herds in Brazil and worldwide (Pinto, 2008).

More recently, this area has received renewed attention, undoubtedly as a consequence of the ability of nisin to inhibit a wide range of mastitis-causing pathogens (Cao et al., 2007). The development of non-antibiotic formulations, such as bacteriocins, has the potential to reduce the dependence on antibiotics for prophylactic therapies in the future.

In short, *L. lactis* subsp. *lactis* PD6.9 displays a good potential for bacteriocin production. Because, *L. lactis* is generally recognized as safe for human health and *L. lactis* PD6.9 was obtained from a food source, its reintroduction in fermented meat should not impose technological problems for consumption of the final product. The bacteriocin produced by *L. lactis* PD6.9 is potentially active against foodborne pathogens, such as *S. aureus* and *L. monocytogenes*. The use of bacteriocin and bacteriocin-producing strains as starters or protective cultures in food preservation will contribute to the safe and wholesome food production. In addition, its bacteriocin showed an inhibitory effect against mastitis-causing pathogens, being attractive to replace antibiotics for prophylactic therapies. *L. lactis* PD6.9 is a new strain, originating from fermented products, presenting good production and desirable characteristics.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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Evaluation of antibacterial effects and phytochemical screening of the aqueous and methanolic extracts of *Hibiscus diversifolius*

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Hibiscus diversifolius **which is widely distributed in Kenya was investigated for its antibacterial effect against *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Staphylococcus aureus*. The leaves stem and root of the plant was extracted using aqueous and methanol as solvents. Phytochemical screening was also carried out to determine the phytochemical constituents present in the various parts of the plants used. The results show that both aqueous and methanolic extracts of the different plant parts had antibacterial activity against the various microbes tested. Phytochemical screening revealed the presence of alkaloids, flavonoids, sterols, saponins, terpenoids and cardiac glycosides while tannins and steroids were lacking in all the extracts.**

Key words: Antibacterial, phytochemicals, aqueous, methanol and extracts.

INTRODUCTION

For a long period, plants have been a valuable source of natural products for maintaining human health and impressive number of modern drugs have been isolated from them; many on their use in traditional medicine (Nascimento et al., 2000; Nair et al., 2005). Currently, it is estimated that over 50% of all modern clinical drugs are of natural products origin (Cordell, 2000; Newman et al., 2003). These drugs are employed in the treatment of both infectious and non-infectious diseases. Infectious diseases remain the leading cause of death and account for one quarter of all deaths in the world (WHO, 1999). To worsen matters, infections due to antibiotic resistant micro-organisms have been on the rise (Pfaller et al., 1998). There is therefore urgent need to come up with new novel antimicrobial agents to combat and curb the spread of these resistant microbes. Higher plants are still poorly explored as sources of new drugs (Hostettman and Terreaux, 2000) and would therefore be a good starting

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point in the search for efficacious novel antimicrobial agents. *Hibiscus diversifolius* is an annual shrub belonging to the family Malvaceae with a wide distribution in Kenya and other parts of the world. Medicinal uses of plants from this family have been reported in traditional folklore medicine and the most frequently cited are antibacterial, antihelminthic and antimalarial. They have reportedly been used in the treatment of cancer, abscesses, bilious conditions, bruises, cough and pneumonia (Olaleye, 2007; Ngari et al., 2010; Agbor et al., 2005). A few members of the genus Hibiscus have received scientific attention. Methanolic extracts of *H. sabdariffa* were demonstrated to have antibacterial activity against a number of selected pathogens (Olaleye et al., 2007). *H. cannabinus* have been investigated for their haematinic property in anaemic rats in addition to its phytochemical constituents (Agbor et al., 2005). *H. diversifolius* has never been scientifically investigated. This study was therefore undertaken to determine the antibacterial effect of this plant and also sought to establish the phytochemicals present in this plant as some of these could be used to explain the observed antibacterial effects if any.

MATERIAL AND METHODS

Plant materials and their collection

Plant materials were collected from Oyugis located in Homa-bay county of Kenya during the month of June, 2011. Leaves, stem and root of *H. diversifolius* were collected. The plant was identified in the herbarium, Department of Botany, Jomo Kenyatta University of Agriculture and Technology (JKUAT) , where voucher specimens were deposited. The plant materials were dried under shade at temperature below 30ºC and pulverized in a hammer mill fitted with a sieve of 0.5 mm pore.

Preparation of methanolic extracts

The ground plant material was extracted twice with methanol as the solvent of extraction. One hundred grams of plant powder was extracted by mixing with 300 ml of methanol. The slurry of solvent and plant powder was stirred and left to stand for 48 h, after which the supernatant was filtered through Whatman® GF/C glass microfiber filter paper and the filtrate concentrated under vacuum at 40ºC in Buchii rotary evaporator. The extracts were then dried in a freeze drier and kept desiccated at 4ºC until use.

Preparation of aqueous extracts

Plant powders' weighing 300 g was boiled for 20 min in 800 ml of distilled water. After cooling to room temperature, the supernatant was decanted, centrifuged at 5400× gravity for 10 min after which the supernatant was filtered through Whatman® GF/C glass microfiber filter paper, frozen at -15ºC and then dried in a freeze drier. The extract was kept desiccated at 4ºC.

Antimicrobial screening of *H. diversifolius* extracts

Antibacterial activities of the plant extracts of *H. diversifolius* were tested by disc diffusion method. Four bacterial strains *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Staphylococcus aureus* were used in this study. The organisms were obtained from a culture collection maintained in the department of Botany, JKUAT. The bacteria were tested for purity by culturing on nutrient agar and maintained on nutrient agar slants.

Preparation of inocula

Stock cultures were maintained on slopes of nutrient agar. Active cultures for experiments were prepared by transferring a loopful of cells from the stock cultures to test tubes of Mueller-Hinton broth (MHB) and reactivated by culturing overnight at 37ºC. Cultures were diluted with fresh MHB and compared with McFarland standard to achieve values corresponding to 2×10^6 colony forming units.

Antibacterial activity of the extracts

Antibacterial activity of the plant extracts was tested by disc diffusion method as described by Mbwambo et al. (2007). Four strains of bacteria were used, Gram negative *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC 27853) and Gram positive *S. aureus* (ATCC 25923), and *B. subtilis* (ATCC 6633). Filter paper discs (Whatman No.1) 6 mm diameter were impregnated with crude extracts. Discs dipped into methanol and distilled water served as negative control. All the bacteria were incubated at 30ºC for 24 h by inoculation into nutrient broth. Sterilized Petri- dishes were inoculated with 0.01 ml of one of the above culture media (10^5-10^6 per ml). Mueller- Hinton agar sterilized in a flask and cooled to 45- 50ºC was distributed by pipette (15 ml) into each inoculated Petri dish and swirled to distribute the medium homogenously. Discs injected with extracts at different concentrations were applied on the solid agar medium by pressing slightly. Standard antibiotic discs of streptomycin (25 ug), tetracycline (100 ug) and gentamycin (10 ug) were also included and tested for their antibacterial activity against test microbes. The treated Petri dishes were placed at 4ºC for 1-2 h and then incubated at 35ºC for 18-24 h. The discs were tested in triplicate. At the end of the period, the inhibition zones formed on the media were measured with a transparent ruler in millimeters.

Phytochemical screening

Test for the presence of compounds present in plant extracts

The methods described by Nanyemi et al. (2005) and Banso and Adeyemo (2006) were used to test for the presence of alkaloids, flavonoids, sterols and steroids, saponins and tannins.

Determination of alkaloids

Extract of each plant sample was separately stirred with 1% hydrochloric acid (HCIL) on a steam bath. The solution obtained was filtered and 1 ml of the filtrate was treated with two drops of Mayer's reagent. The two solutions were mixed and made up to 100 ml with distilled water. Turbidity of the extract filtrate on addition of Mayer's reagent was regarded as evidence for the presence of alkaloids in the extract.

Determination of flavonoids

To 1 ml of each plant extract in a test tube was added a small piece (2mm strip) of magnesium ribbon followed by drop wise addition of

Table 1. Antibacterial effects of aqueous extracts of leaf, stem and root of *H. diversifolius* against test microbes in mm after 3 repeats.

Extract	Microbe	Concentrations (mg/ml)				
		0.01	0.1	1	10	100
Leaf	*E. coli*	9.0±0.3	8.9±0.3	9.0±0.3	8.1±0.3	9.0±0.3
	P. aeruginosa	11.0±0.2	11.0±0.1	10.0±0.2	9.0±0.3	8.9±0.3
	B. subtilis	10.0±0.1	10.0±0.2	10.1±0.2	9.0±0.1	8.0±0.1
	S. aureus	10.1±0.1	10.4±0.7	10.0±0.2	9.0±0.1	9.8±0.5
Stem	*E. coli*	8.0±0.2	8.1±0.2	10.0±0.2	8.1±0.2	7.1±0.1
	P. aeruginosa	9.0±0.3	10.0±0.1	10.0±0.1	10.0±0.2	9.1±0.2
	B. subtilis	9.0±0.2	10.0±0.2	10.0±0.1	9.0±0.2	8.0±0.2
	S. aureus	10.0±0.2	10.0±0.2	9.9±0.2	9.0±0.2	9.1±0.2
Root	*E. coli*	9.0±0.2	9.0±0.3	8.1±0.3	10.1±0.2	10.1±0.2
	P. aeruginosa	9.0±0.2	10.1±0.2	9.0±0.3	10.1±0.2	9.0±0.2
	B. subtilis	8.1±0.3	9.1±0.3	10.1±0.2	9.0±0.2	8.1±0.2
	S. aureus	10.0±0.3	9.0±0.2	9.1±0.2	10.1±0.1	8.9±0.2

concentrated hydrochloric acid. Development of pink or magenta red colors indicated the presence of flavonoids.

Determination of sterols and steroids

One milliliter of extract was put into a test tube in which 0.5 ml sulfuric acid, acetic anhydride and chloroform in similar amount was added. A red coloration indicated presence of sterols while a green color indicated presence of steroids.

Determination of saponins

milliliter of each extract under test was put into a test tube and 50 ml of tap water added. The mixture was then shaken vigorously. Foaming which persisted on warming was taken as an evidence for the presence of saponins but this was subjected to further confirmatory test. This involved dissolving 1 ml of the extract in carbon tetrachloride to which 4 drops of concentrated sulphuric acid was added to the mixture. A blue, green, or red color accompanied by a pink ring confirmed presence of saponins.

Determination of tannins

Ethanolic extract of each sample was separately stirred with 10 ml of distilled water and then filtered. To the filtrate was added two drops of 5% iron III chloride ($FeCl_3$) reagent. Blue- Black or blue green coloration was taken as an indication of the presence of tannins.

Determination of cardiac glycosides

5 ml of extracts were treated with 2 ml of glacial acetic acid containing one drop of ferric chloride solution. This was under laid with 1 ml concentrated sulphuric acid. A brown ring at the interface indicates deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring while in the acetic acid layer a greenish ring may form.

Determination of terpenoids

5 ml of each extract was mixed with 2 ml of chloroform, and 3 ml concentrated sulphuric acid. Formation of a reddish brown coloration at the interface was considered a positive test for the presence of terpenoids.

RESULTS

The results of the antibacterial effect of the different concentrations of the aqueous extracts of leaf, stem and roots of *H. diversifolius* are given in Table 1. These extracts of the different plant parts exhibited antibacterial activities against the selected test microbes with zones of inhibition ranging from 8 to 11 mm. For all the aqueous extracts, the antibacterial effect did not differ much between the various concentrations used as the zones of inhibitions formed were almost of the same size regardless of the concentrations used. There were no differences in the antibacterial effects of the aqueous extracts on both the Gram positive and Gram negative as determined from the zones of inhibitions formed.

The results of the different concentrations of methanolic extracts of leaf, stem and root of *H*. *diversifolius* are given in Table 2. The methanolic extracts of the different plant parts demonstrated antibacterial effect against the different microbes used in this study with zones of inhibition ranging from 6.9 to 12 mm. The antibacterial effect of the different plant parts did not differ much from each other as can be deduced from the resulting zones of inhibitions formed. Varying concentrations of the different extracts did not produce big differences in their antibacterial effect. Again the antibacterial effect of the methanolic extracts against Gram positive and Gram negative organisms tested were almost similar. From the

Table 2. Antibacterial effects of methanolic extracts of leaf, stem and root of *H. diversifolius* against test microbes in mm after three repeats.

Extract	Microbe	Concentrations (mg/ml)				
		0.01	0.1	1	10	100
Leaf	*E. coli*	10.1±0.2	10.2±0.2	10.0±0.3	9.1±0.2	7.0±0.2
	P. aeruginosa	10.0±0.2	12.0±0.1	10.0±0.2	9.0±0.2	8.1±0.2
	B. subtilis	8.1±0.2	10.1±0.2	8.0±0.1	10.3±0.8	8.0±0.2
	S. aureus	10.1±0.2	8.9±0.2	12.0±0.2	9.0±0.3	7.0±0.2
Stem	*E. coli*	10.0±0.2	9.9±0.2	9.9±0.4	8.1±0.2	8.9±0.2
	P. aeruginosa	10.0±0.2	6.9±0.2	8.1±0.3	8.0±0.3	7.1±0.2
	B. subtilis	10.0±0.3	9.0±0.2	8.9±0.2	10.3±0.2	9.9±0.2
	S. aureus	9.1±0.2	7.0±0.2	8.1±0.2	9.9±0.2	7.9±0.2
Root	*E. coli*	10.0±0.3	10.1±0.2	10.0±0.2	9.1±0.3	7.1±0.2
	P. aeruginosa	10.1±0.2	12.1±0.3	10.0±0.3	9.1±0.3	8.1±0.2
	B. subtilis	8.0±0.2	10.4±0.8	7.9±0.2	10.0±0.2	8.1±0.2
	S. aureus	10.1±0.2	9.4±0.3	12.0±0.2	9.0±0.3	7.0±0.2

Table 3. Phytochemical components of leaf, stem and root of *Hibiscus diversifolius* extracted with aqueous and methanolic solvents.

Extract	Phytochemical							
	Alkaloid	Flavonoid	Sterol	Steroid	Saponin	Tannin	Terpenoid	Cardiac glycoside
Methanolic extract								
Leaf	+	+	+	-	+	-	+	+
Stem	+	+	+	-	+	-	+	+
Root	+	+	+	-	+	-	+	+
Aqueous extract								
Leaf	+	+	+	-	+	-	+	+
Stem	+	+	+	-	+	-	+	+
Root	-	-	-	-	-	-	-	+

two tables, the aqueous and methanolic extracts seem to have similar antibacterial activities as the zones of inhibitions produced were almost of similar sizes.

The result of phytochemical screening of the aqueous and methanolic extracts of leaf, stem and root of *H. diversifolius* are provided in Table 3. All the methanolic extracts of the 3 plant parts were found to contain all the phytochemical compounds tested for except steroids and tannins. For aqueous extracts of the leaf, stem and root of *H. diversifolius*, phytochemical screening revealed that the leaf and stem had similar compounds present in them. All the phytochemicals tested for except steroids and tannins were present. This composition was similar to that of the methanolic extracts of leaf, stem and roots. A notable exception was seen with the aqueous root extract which lacked all the phytochemicals except for cardiac glycosides but showed similar antibacterial effects as the other extracts.

DISCUSSION

The present study has demonstrated that all the aqueous and methanolic extracts of *H. diversifolius* had antibacterial activity against all the microbes tested and that the different plant parts had almost similar antibacterial effect against both the Gram positive and Gram negative organisms used in this particular investigation. It has been published that various plant extracts have been demonstrated to possess antibacterial activity against microbial pathogens (Mahesh and Satish, 2008; Balakrishnan et al., 2006; Mehrgan et al., 2008; Mandal et al., 2000). The antimicrobial activity observed could be due to the varied phytochemicals present. Indeed, several metabolites from herb-species such as alkaloids, tannins, saponins and steroids have previously been associated with antimicrobial activity (Leven et al., 1979). It is necessary to identify the phytochemical components of local medicinal plants because the presence or absence of certain phytochemicals could be used to explain some of the biological activity of certain plant extracts observed. The antibacterial activity of the methanolic extracts of the leaf, stem and root could be attributed to the presence of alkaloids, flavonoids, steroids, saponins, terpenoids and cardiac glycosides.

Phytochemical screening of the aqueous extracts of the leaf and stem of *H. diversifolius* showed that all the phytochemical constituents except steroids and tannins were present and therefore the antibacterial effects could also be associated with these compounds. Phytochemical compounds present in these two aqueous extracts were similar to those present in all the methanolic extracts. It is therefore not surprising that all these extracts had almost similar antibacterial effect as could be discerned from the sizes of the zones of inhibitions formed. The aqueous root extract *of H. diversifolius* lacked all these phytochemicals present in all other extracts except for the presence of cardiac glycosides only. Yet surprisingly, it demonstrated almost similar antibacterial effect comparable to other extracts. Its antibacterial activity can only be attributed to cardiac glycosides as it was the only compound whose presence was revealed by phytochemical screening. Aboaba et al. (2006) has reported that many plants contain toxic glycosides which can get hydrolysed to release phenolics which are toxic to microbial pathogens. It is also possible that there are other compounds besides the ones tested for could be contributing to the antibacterial activity of this particular extract. Indeed, Astal et al. (2005) reported that the water extracts of *Salvadora persica* roots and stems contained potential antimicrobial anionic components such as chloride, sulfate, thiocyanate and nitate. It is possible that the same compounds may have been present in this particular extract and contributed to the antibacterial effect seen.

Indole quinolone alkaloids, glycoalkaloids, berberine type alkaloids, indole quinolizidine alkaloids have been reported to be active against a range of Gram positive and Gram negative bacteria (Iwu et al., 1999). Flavonoids have been found to show *in vitro* antimicrobial activity against a wide range of bacteria. Their activity has been attributed to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell wall (Cowan, 1999). There is no information on the antibacterial effects of plant steroids and sterols. Saponins have been reported to possess antifungal activity (Iwu, 2000). Terpenoids are terpenes to which additional elements such as oxygen have been added (Cowan, 1999). Terpenes and terpenoids have been found to possess antimicrobial activity (Mendoza et al., 1997; Amara et al., 1998). The mechanism of action of terpenes on microbes is not yet fully understood, but it is speculated to involve membrane disruption by the lipophilic compounds (Mendoza et al., 1997). Cantrell et al. (2001) investigated a series of terpenoids for their antimicrobial effects and found out that the more lipophilic compounds were significantly more antibacterial than their more polar analogues. Tannins are a large group of polyphenolic compounds that are subdivided into two groups; hydrolysable and condensed tannins. A wide range of anti-infective actions have been assigned to them (Haslam, 1989). It has been postulated that the anti microbial mode of action for tannin may thus be related to their ability to inactivate microbial adhesins, enzymes and cell envelope transport proteins (Cowan, 1999).

There is evidence that tannins may directly inactivate micro-organisms due to their ability to bind proteins and metals, and also to inhibit the growth of micro-organisms through substrate and metal ion deprivation (Brownlee et al., 1989). The extent to which these phytochemicals present in these extracts of *H. diversifolius* contribute to its antibacterial effect cannot be discerned. These could be exerting their effect through additive or synergistic action of several com-pounds acting at a single or multiple target sites associated with physiological process.

Conclusion

The aqueous and methanolic extracts of the different plant parts of *H. diversifolius* have demonstrated similar antibacterial effects. Except for the aqueous root extract, all other extracts had similar phytochemical constituents. The result suggests that some of the extracts of this plant should be investigated further as they could serve as source of drugs useful in the chemotherapy of some microbial infections.

Conflict of Interest

The author(s) have not declared any conflict of interest.

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