

Original Research Article

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Development of Herbal-Silver Nano Composite (HSNC): Antibacterial Evaluation and Investigation of *fnb A* and *fnb B* Genes Coding Fibronectin-binding Proteins in *Staphylococcus aureus*

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ABSTRACT

A novel composite synthesized using silver nanoparticles with *Terminalia chebula* extracts was selected as the primary objective of this present research. Also an investigation on the virulence genes, *fnbA* and *fnbB* that regulate the production of the fibronectin binding proteins in *S. aureus* using polymerase chain reaction was studied. Silver nanoparticles was synthesized and a novel composite was developed using medically significant *Terminalia chebula* extracts in three different ratios (1:1, 1:2 and 2:1). Antibacterial activity of the developed herbal-silver nanocomposite (HS_{NC}) against the seven *Staphylococcus aureus* strains was determined. The presence of genes encoding adhesin specific fibronectin-binding proteins of *fnbA* and *fnbB* in the selected *Staphylococcus aureus* strain-7 was investigated using standard polymerase chain reaction method. The obtained inhibitory clear zones range from 38mm to 49mm for all the ratios against all the test strains. Maximum inhibition clear zone (ICZ) of 49mm was observed for three different ratios, 1:1, 1:2 and 2:1 against test strains- 1, 5 and 7 respectively; and the lowest of 38mm for all the ratios against test strains-1, 2, 3 and 4 respectively. In the selected strain (*S. aureus* 7), both *fnbA* and *fnbB* genes were detected. This was evident and proved by comparing the electrophoretic DNA patterns of the reference strain, *Staphylococcus aureus* NCTC 8325. The obtained results were considered to be as a preliminary experiment for the development of a novel pharmacological product in future.

Keywords

Silver ions,
Composite,
Fibronectin,
Inhibition clear
zone, *fnbA* and
fnbB.

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Introduction

Foot ulcers are an increasing problem in patients with *Diabetes mellitus* and infection is a frequent complication that actually constitutes the most common cause of hospitalization in diabetic patients, often related to lower-extremity amputation (Spichler *et al.*, 2015). The lifetime risk of developing foot ulcer in diabetic patients is

about 25%. The high rate of infection in such ulcers is considered as an important cause of amputation in 25–50% of diabetics and therefore is associated with morbidity and mortality in a large number of individuals (Mendes *et al.*, 2012). Diabetic foot infections (DFI) are often polymicrobial and can be caused by several pathogens,

mainly Gram positive bacteria, being *Staphylococcus* the most predominant bacterial genus. *Staphylococcus* is a frequent commensal bacterium of human skin and mucosa, being one of the major causes of infections in humans, ranging from minor skin infections to severe infections such as septicaemia, endocarditis and osteomyelitis (Zenelaj *et al.*, 2014).

The colonization and infection is mainly due to the presence of different virulence determinants including toxins, tissue degrading enzymes and immune evasion factors. Several virulence genes are implicated in biofilm formation, like *icaA* and *icaD*, responsible for the biosynthesis of polysaccharide intercellular adhesion (PIA) molecules, containing N-acetylglucosamine, the main constituent of the biofilm matrix in the accumulation phase (Cos and Tote, 2010).

The staphylococcal surface adhesins named as “microbial surface components recognizing adhesive matrix molecules” (MSCRAMMs) enable bacteria to bind to the fibronectin, fibrinogen, and collagen of the host. In staphylococci, binding to proteins such as fibrinogen, elastin, and fibronectin is mediated by adhesins, which are named FNBP A and FNBP B and are under the control of *fnbA* and *fnbB* genes (Nashev). Fibronectin binding proteins were investigated in both methicillin susceptible and resistant *S. aureus* strains isolated from patients with staphylococcal infections such as osteomyelitis and skin and soft tissue infections.

Presence of the *fnbA* gene was reported as approximately 100% and for the *fnbB* gene this proportion was reported to be between 0% and 98% in clinical isolates (Arciola *et al.*, 2005). The aim of the present study was to investigate the genes that regulate the

production of the fibronectin binding proteins in the *S. aureus* strains using polymerase chain reaction.

There has been an increasing incidence of multiple resistances in human pathogenic microorganisms, largely due to the indiscriminate use of commercial antimicrobial drugs commonly employed in the treatment of infectious diseases (Alleroand Afolayan, 2006). The development of bacterial resistance to presently available antibiotics has necessitated the search for new antibacterial agents (Parekh and Chanda, 2007). Therefore there is a need to develop alternative antimicrobial drugs for the treatment of infections obtained from various sources such as metal nanoparticles and medicinal plants.

Nanotechnology is an emerging science and with growing use particularly in developing new materials at nanoscale levels (Albrecht *et al.*, 2006). From among different types of available nanomaterials, nanosilver is proved to be most effective material which has good antimicrobial properties against bacteria, viruses and other eukaryotic microorganisms (Gong *et al.*, 2007). Silver ions have long been known to exert strong inhibitory and bactericidal effects as well as to possess a broad spectrum of antimicrobial activities (Berger *et al.*, 1996). Silver ions are internalized and react with thiol groups of cellular proteins that lead to uncoupling of ATP synthesis from respiration, loss of proton motive force and interference with phosphate efflux system. At levels of millimolar silver nanoparticles induce the detachment of the cell wall membrane from the cytoplasm, with the possible release of intracellular content, DNA condensation and loss of replicative capacity (Marambio-Jones and Hoek, 2012).

Numerous studies have been conducted with the extracts of various plants, screening antimicrobial activity as well as for the discovery of new antimicrobial compounds (Parekh and Chanda, 2007). The efforts of scientists in establishing plants with promising antimicrobial property is yielding fruitful results as a number of plants with high antimicrobial property have been elucidated (Dash *et al.*, 2005). *Terminalia chebulais* a medium size to large tree, up to 25-30 cm tall with many spreading branches with pale greenish, gray and smooth bark, expontanea on India, Ceylon, Burma, Malayan peninsula, Siam an cultivated in Pakistan. In English is known as black myrobalan or chebulicmyrobalan. Two varieties of this species are recognized - *Terminalia chebula* var. *chebula* and *Terminalia chebula* var. *tomentella* (Malckzadeh *et al.*, 2001).

It is called the “king of medicines” and is the most important medicinal plant in the Ayurvedic materiamedica due to its general use. The fruit of this species is one of the constituents of Triphala, a popular traditional herbal preparation used also for different chronic diseases like diabetes (Bag *et al.*, 2011). The dried ripe fruit of *T. chebula* is the plant part used in Indian traditional medicine as homeostatic, antitussive, laxative, diuretic and cardiotoxic agent, and to treat chronic ulcers and wounds.

Based on the significant antibacterial actions of silver and *Terminalia chebula*, a novel herbal-silver nanocomposite was developed in the present research. Antibacterial activity of the nanocomposite against five different strains of *Staphylococcus aureus* was determined under standard *in vitro* conditions. Also, investigation on the virulence genes, *fnbA* and *fnbB* that regulate the production of the fibronectinbinding

proteins in *S. aureus* using polymerase chain reaction was studied.

Materials and Methods

In the present research, development of herbal-silver nanocomposite and its antibacterial activity; identification of virulence genes were carried out in PG and Research Department of Microbiology, GRD College of Science and Commerce, Coimbatore, India, from February 2016 to September 2016. Medicinal herb, *Terminalia chebulawas* collected and authenticated from Tamil Nadu Agricultural University, Coimbatore, India.

Solvent extraction of collected herbs (Tiwari *et al.*, 2006)

Extraction was carried out by dissolving 6 grams of the Selected herbal powder *Terminalia chebulain* 100ml of 80% methanol, kept overnight under shaking condition. Then the extract was filtered using Whatmann no.1 filter paper, filtrate was collected and evaporated at room temperature.

Development of herbal nanoparticles (Moradhaseli *et al.*, 2013)

The Nanoparticles were synthesized by using 50ml of herbal methanolic extract *Terminalia chebula* Initially 125ml of sodium alginate (base solution) (3.35mg / ml) was prepared, followed by 75ml of calcium chloride (3mg / ml) was prepared. The calcium chloride (CaCl₂) solution was added a drop wise into sodium alginate solution with constant stirring at 1500 rpm for 30minutes at room temperature. Then the herbal extract was added to the mixture very carefully drop wise to the above solution with constant stirring for 45-60 minutes. The reaction mixtures were kept undisturbed for

overnight. After incubation the uppermost layer is discarded and the pellet was collected and characterized for further processing.

Preparation of Silver nanoparticles

About 20 g of the PEG was dissolved in 1 liter of the RO water before being heated up to 50 degree Celsius. The solution was allowed to be stirred for another 1 hour to ensure all the PEG was completely dissolved to form a homogeneous solution. Aqueous PEG solution obtained was then filtered to remove impurities, if any. Silver nitrate solution prepared using 0.5 g of silver nitrate salt was added into the PEG solution prepared under a constant stirring rate and at constant temperature of 50 degree Celsius. pH of the solution was not controlled. The solution was continuously stirred for 1 hour to complete the chemical reactions. After the formation of the particles, the solution was filtered through the filter paper to separate the particles from the mother solution. Particles obtained were rinsed with RO water several times before it was rinsed again using ethanol. The particles were dried in oven at 60°C for overnight.

Synthesis of antimicrobial herbal-silver nano composite

Herbal and silver nanocomposites were prepared in four different combinations (1:1, 1:2, 2:1, 2:2). Single strength and double strength concentration of herbal and silver nanoparticles was used to attain these ratios. For 1:1 herbal-silver nano composites, 100 mg of herbal nanoparticles were dispersed in 1 ml of sterile distilled water was added drop wise to 100mg nano silver solution. Herbal solution was added at the rate of 1ml per minute. Similar procedure was carried out at controlled

condition for the other ratios. All prepared antimicrobial composites at different ratio were further termed as HS_{NC} (herbal-silver nano composites).

Antibacterial activity of herbal-silver nanocomposite HS_{NC} against the test pathogens

The antimicrobial activity of herbal-silver nanocomposite HS_{NC} was tested using a standard agar diffusion test against seven wound pathogenic strains of *Staphylococcus aureus* (named as strain-1, 2, 3, 4, 5, 6 and 7). Nutrient agar plates were prepared by pouring 15 ml of media into sterile Petri dishes. The plates were allowed to solidify for 5 minutes and 0.1% inoculum was swabbed uniformly and allowed to dry for 5 minutes. The sterile cotton wound gauze materials with the diameter of 2.0 ± 0.1 cm after impregnating in the herbal-silver nanocomposite HS_{NC} solution was placed on the surface of medium and the plates were incubated at 37 °C for 24hours. At the end of incubation, the zone of inhibition formed around the material was measured in millimetres and recorded. The organism showing maximum inhibitory zone was further selected for the identification of virulent gene studies using standard molecular biology methods.

Investigating the presence of genes encoding adhesin specific fibronectin-binding proteins of *fnbA* and *fnbB* using standard polymerase chain reaction method

One organism was selected from the antibacterial activity test based on the maximum inhibition zone produced. The selected organism was inoculated in Columbia Agar + 5 % sheep blood. Plates were incubated at 37 °C for 24 h. Rapid DNA extraction was performed by

suspending four to five bacterial colonies in 100 μ L of TE (10 mM Tris, 1 mM EDTA, pH 7.8) buffer and heating to 97 °C for seven min. After centrifugation at 15 000 g for five min, supernatant was collected and stored at -20 °C for subsequent PCR screening.

The presence of virulence determinants was evaluated by PCR amplification using two different primers. Genes encoding adhesin specific fibronectin-binding proteins of *fnbA* and *fnbB* was amplified using the specific primers developed under standard ambient conditions (Table-1). Commercial NZYDNA ladder VI was used as a molecular weight marker. The amplification reaction was performed with a thermal cycler (ABI2720) and the PCR amplicons were resolved by electrophoresis using 0.5X Tris-Borate-EDTA (TBE) buffer in a 2 % agarose gel (Sigma-Aldrich) and the gels visualized by transillumination under UV.

Results and Discussion

Antibacterial activity of herbal-silver nanocomposite HS_{NC} against the test pathogens

Antibacterial activity of herbal-silver nano composite (HS_{NC}) developed in the present study was expressed as inhibition clear zone (ICZ) against all the test strains of *Staphylococcus aureus* (Strain-1, 2, 3, 4, 5, 6 and 7). Different significant factors like herbal drug and metal nanoparticles in the composite highly influenced the inhibition of the growth of bacteria. From Table-2, it was evident that no significant changes in the inhibition clear zone (ICZ) were observed for any of the composite ratios specifically. All the four different ratios produced good ICZ against all the test strains indicating the mode of action of prepared composite on the bacteria. The

obtained inhibitory clear zones range from 38mm to 49mm for all the ratios against all the test strains. Maximum ICZ of 49mm was observed for three different ratios, 1:1, 1:2 and 2:1 against test strains- 1, 5 and 7 respectively; and the lowest of 38mm for all the ratios against test strains-1, 2, 3 and 4 respectively. Over all strain 7 showed more inhibition clear zones against all the four ratio of nanocomposite (Fig 1). Hence this strain was selected for further studies.

Antimicrobial efficacy of the HS_{NC} coated wound dressing materials has a characteristic feature of synergism. It was clearly understood from their mode of action on bacterial cell components. Silver nanoparticles increase cell membrane permeability and subsequently penetrate into cells, free radicals produce oxidative stress in reactive oxygen species (ROS) resulting in membrane damage and DNA (Marambio Jones and Hoek, 2012). Feng *et al.*, (2000) emphasized that the bactericidal effects observed in this study might have been influenced by the release of silver ions. Silver ions cause the release of K⁺ ions from bacteria; thus, the bacterial plasma or cytoplasmic membrane, which is associated with many important enzymes and DNA, is an important target site of silver ions (Schreurs and Rosenberg, 1982). In addition, silver ions can interact with nucleic acids (Rahnand Landry, 1973); they preferentially interact with the bases in the DNA rather than with the phosphate groups, although the importance of this mechanism in terms of their lethal action remains unclear (Zavriev *et al.*, 1979).

Like the mode of action of silver ions which cross links within the nucleic acid strands, resulting in the disorganized structure, ofloxacin and ornidazole also targets the type II DNA topoisomerase, DNA gyrase and DNA topoisomerase IV of bacteria.

These hetero-tetrameric enzymes manipulate DNA topology by introduction of transient double-stranded breaks in bound DNA (G-segment) through which a second DNA fragment (T-segment) that are ultimately lethal to the cell (Drlica and Zhao, 1997).

The bactericidal effect of metal nanoparticles has been attributed to their small size and high surface to volume ratio, which allows them to interact closely with microbial membranes and is not merely due to the release of metal ions in solution (Theivasanthi and Alagar, 2010). Alamet *et al.*, (2009) suggests that the plant extract

certainly posses some chemical constituents with antimicrobial properties. *Terminalia chebula* used in the composite thus contains several antibacterial compounds like triterpenoids and hydrolysable tannins. These phytochemical compounds contain significant chemical constituents such as chebulic acid, chebulagic acid, chebulinic acid, corilagin, punicalagin, chebulanin, terchebulin and gallic acid (Upadhyay *et al.*, 2014). Based on this proposed mode of action of silver nanoparticles on the bacterial membrane, in the present study a novel nanocomposite with herbal and metal formulation was developed.

Table.1 Forward and reverse primers of *fnbA* and *fnbB*

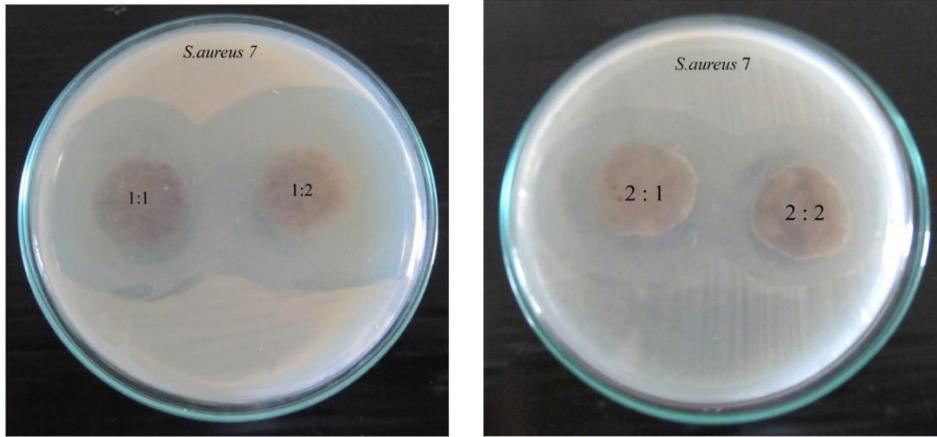
S.No	Target genes	Primer type	Nucleotide sequence
1	<i>fnbA</i>	Forward	5' – CCACCTGGGTTTGTATCTTCTTC – 3'
	<i>fnbA</i>	Reverse	5' – GATTACCACACAGCTATAGATGGTG – 3'
2	<i>fnbB</i>	Forward	5' – CGTGACCATTTTCAGTTCCTAAACC – 3'
	<i>fnbB</i>	Reverse	5' – GATACAAACCCAGGTGGTGG – 3'

Table.2 Antibacterial activity of herbal-silver nanocomposite HSNC against the test pathogens

S. No	Nanocomposite	Ratio	Sample	Zone of inhibition (mm)						
				<i>Staphylococcus aureus</i>						
				1	2	3	4	5	6	7
		1 : 1		49	47	38	45	44	45	47
1	<i>Terminalia chebula</i> -Ag Nanocomposites (HS _{NC})	1 : 2	100% Cotton gauze	38	42	42	44	49	47	48
		2 : 1		45	41	45	38	48	45	49
		2 : 2		41	38	41	43	44	47	48

HS_{NC}: Herbal (*Terminalia chebula*) and silver nanocomposite prepared in four different ratio exhibits good antimicrobial activity against all test strains.

Fig.1 Antibacterial activity of herbal-silver nanocomposite HSNC against the test pathogen (*S. aureus* strain 7)



HS_{NC}: Herbal (*Terminalia chebula*) and silver nanocomposite prepared in four different ratio exhibits good antimicrobial activity against the test strain-7 (*S. aureus* 7)

Fig.2 Investigating the presence of genes encoding adhesin specific fibronectin-binding proteins of *fnbA* and *fnbB*



Fig.2(a) Extraction of Genomic DNA from Bacterial sample using the Bacterial Genomic DNA Isolation Kit (RKN15)

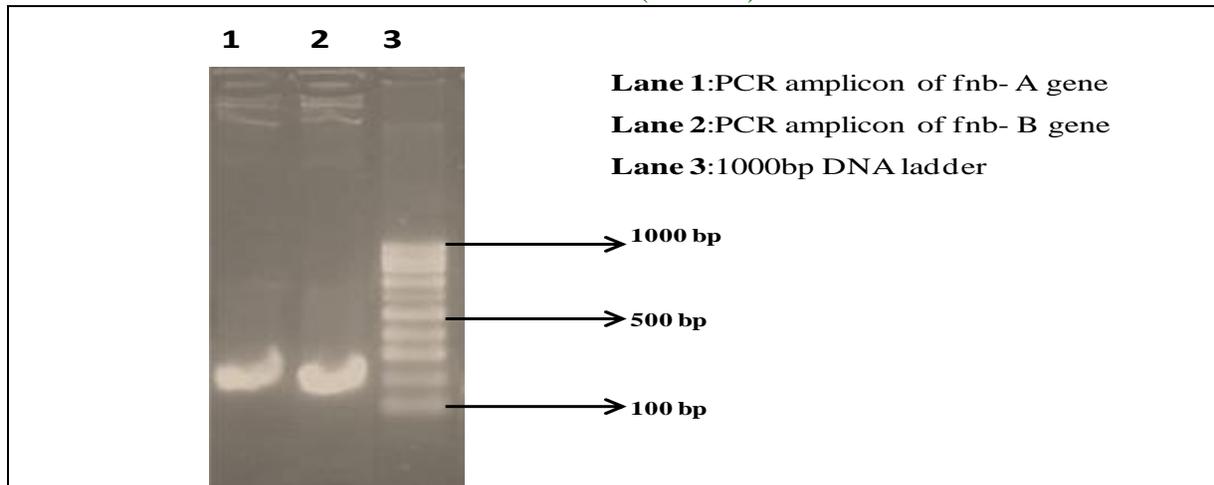


Fig.2(b): PCR amplicons for *fnbA* and *fnbB* loaded on agarose gel

The composites were tested for its antimicrobial efficacy against medical significant coagulase positive *Staphylococcus aureus*. Bisi-Johnson *et al.*, (2005) studied that *S. aureus* resistant against first line antibiotics. The organisms were found to be potent pus producing pyogenic pathogens which is also considered as an etiological agent in diabetic foot ulcer cases. The infection due to these pathogens may leads to foot amputation in similar patients. The developed antimicrobial nanocomposite was aimed to prevent such critical cases with diabetic foot ulcers.

Investigating the presence of genes encoding adhesin specific fibronectin-binding proteins of *fnbA* and *fnbB* using standard polymerase chain reaction method

The prevalence of virulence genes *fnbA* and *fnbB* that regulate the production of the fibronectin binding proteins in the *S. aureus* strains using polymerase chain reaction was selected as the primary objective of this study. In the selected strain (*S. aureus* 7), both *fnbA* and *fnbB* genes were detected (Fig 2). This was evident and proved by comparing the electrophoretic DNA patterns of the reference strain, *Staphylococcus aureus* NCTC 8325 which contains both *fnbA* and *fnbB* genes in their chromosomal DNA. *Staphylococcus aureus* is a pathogen capable of causing numerous community and hospital acquired infections. It expresses “microbial surface components recognizing adhesive matrix molecules” (MSCRAMMs), which promote colonization of host tissue and contribute to infection (Nashev *et al.*, 2004).

In a study conducted by Mishaan *et al.*, (2005) the authors studied community-acquired, methicillin-resistant *S. aureus*

infections and showed that infections were caused by microorganisms belonging to 3 different clones. They detected *fnbA* in all strains while the rate of *fnbB* was 99%, 43%, and 0% in the 3 clones, respectively. In another study focusing on strains isolated from soft tissue infections, the rate of *fnbA* was reported as 76.1% (Zmantar *et al.*, 2008). These literature survey supports that as per the aim of the present study, the genes encoding the virulence factors play a role in the pathogenesis of community acquired musculoskeletal and skin/soft tissue infections.

In conclusion, the persistent increase in multidrug resistant strains compels the search for more potent new antibiotics. Thus there is a need for a continuous search for new effective and affordable antimicrobial drugs. The results of present study signify the potentiality of herbal-silver nanocomposite as a therapeutic agent which may provide leads in the ongoing search for antimicrobial botanicals. Even though many metal nanoparticles coated wound dressing materials were commercially available, the present research revealed the pharmacological application of a novel metal and herbal nanocomposite (HS_{NC}). As a future perspective, the HS_{NC} shall be prepared along with other biomaterials like collagen to form a human skin equivalent (HSE) material by a continuous layer by layer assembly process. Thus the present investigation was proved to be a preliminary experiment for the development of a novel pharmacological product in future.

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