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Research Article
Preparation of a Metal and Herbal Nanocomposite (HC_{NC}): Coating onto Wound Dressing Materials and Evaluating its Pharmacological Properties Like Antimicrobial Activity and Biocompatibility

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Abstract

Background: Copper oxide nanoparticles with extracts of *Smilax china* synthesized as a nanocomposite (HC_{NC}) was studied in the present study. Wound dressing materials, bamboo and cotton were coated with HC_{NC} and its pharmacological properties like antimicrobial activity was determined against diabetic wound pathogens, *Staphylococcus aureus*. **Methodology:** Four different ratios (1:1, 1:2, 2:1 and 2:2) of HC_{NC} were prepared separately and coated onto bamboo and cotton materials using a standard slurry-dipping technique. All the coated materials were investigated for its antibacterial activity, topographical analysis using scanning electron microscope and biocompatible properties using a standard HET-CAM assay. Four different ratios of HC_{NC} coated onto bamboo and cotton materials showed good antibacterial activity against all the seven strains of *Staphylococcus aureus*. Inhibitory Clear Zones (ICZ) for different ratios of HC_{NC} coated bamboo and cotton ranges from 40-52 and 38-51 mm, respectively when tested against all the seven strains. **Results:** During HET-CAM assay no irritation endpoints were observed for the wound dressing materials coated with HC_{NC}, which determined its biocompatible property. **Conclusion:** The present study was carried out on an understanding of the mechanisms underlying this process has salient clinical application, as wound healing may be pharmacologically modulated to enhance repair of any tissue injury.

Key words: Nanocomposite, wound dressing material, pharmacological application, *Staphylococcus aureus*, HET-CAM

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Delay in wound healing was mainly due to bacterial colonization and infection in many hospitalized patients. Topical antimicrobials used to treat wounds are the chemicals that control the microbial growth at wound site[1]. Even though systemic and topical antibiotics play a crucial role in treating open wounds, still continuous use of chemical antimicrobials results in increasing numbers of antibiotic resistant organisms. These significant organisms are methicillin-resistant *Staphylococcus aureus*, *Pseudomonas aeruginosa* and vancomycin-resistant *Enterococcus faecalis*[2]. For some patients, such as those with compromised arterial flow and chronic odema, the use of systemic antibiotics may have poor efficacy, therefore, the treatment of chronic wounds with superficial, local wound infection is difficult[3].

Antimicrobial dressings can be used on acute or chronic wounds which are critically colonised, or when local and/or systemic infection is already compromising the wound or could compromise wound healing. When choosing an appropriate wound dressing it is vital to assess whether the wound is colonised, critically colonised or infected[4].

Delay in wound healing in the patients may be due to different factors. One such unknown reason was revealed to be as multiplication of bacteria at wound site. This is clinically termed as critical colonization at open wound site. This critical colonization is considered to be as a challenge to majority of medical practitioners. In order to reduce the bacterial bioburden in patients, topical antiseptic wound dressing is considered significant[5].

Cross-linked polymerized dextran containing iodine wound dressing is commercially available for treating open wounds in hospitalized patients. As the dressing hydrates in the moist wound environment, elemental iodine is released to exert an antimicrobial effect and to interact with macrophages to produce Tumour Necrosis Factor (TNF) and interleukin (IL-6), which indirectly influence wound healing. Activated charcoal dressings provides moist wound healing environment for autolytic debridement as added advantage. The dressing materials effectively absorb bacteria, prevent the formation of exuberant granulation tissue and reduces wound odour[6]. Another commercially available silver chloride coated nylon wound dressings releases antimicrobial silver ions, which are found effective in killing equine pathogens like *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Streptococcus equi* and *Staphylococcus aureus* under *in vitro* conditions[7].

Human orthopedic and soft tissue post surgery was treated with another commercial wound healing antibiotic impregnated collagen sponges[8]. The dressing exerts its hemostatic effect by causing the adhesion and aggregation of platelets and certain bridge proteins such as fibronectin. This type-1 bovine collagen loaded with gentamicin has hemostatic effect; controlled release of gentamicin due to passive diffusion and breakdown of collagen by macrophages from the matrix prevents wound infections[9].

In the present study, to reduce the colonization of *Staphylococcus aureus* from diabetic foot ulcer cases, one such antimicrobial dressing material coated with *Smilax china* (herb) and copper oxide nanocomposite (Herbal copper nanocomposite-HC_{NC}) was investigated. Under controlled *in vitro* conditions seven different strains of *Staphylococcus aureus* were investigated for its sensitivity pattern against a developed antimicrobial dressing material. The composites were prepared in nano structures; containing herbal and metal composition. Thus, the present study was carried out on the concept of Colella *et al.*[10] describing that, an understanding of the mechanisms underlying this process has salient clinical application, as wound healing may be pharmacologically modulated to enhance repair of any tissue injury.

Smilax china is a novel and medical significant plant species selected based on the perception of Ahmad and Beg[11]. There is no much information available about the medical significance of this plant; infact another species of this plant provided more antimicrobial and analgesic properties. According to Na *et al.*[12], the bioactive compounds found in 1 species may contain active compounds of another species of similar genus.

Copper in oxide form is synthesized by wet chemical process. Copper nano metals used in this research was selected based on the concepts described by Theivasanthi and Alagar[13]. Copper in nanocrystal form applied as antibacterial and antifungal agent after incorporating as nanocoating layers in plastics and textiles. Thus, nanomaterials prepared using nano copper particles plays a crucial role in the field of nanomedicine, bionanotechnology. This gained more interest in the development of nanotoxicology research in the near future.

To reduce pathogenic bacteria like to cause potential deadly infections was reported to be controlled by using copper as an antimicrobial agent. This was also approved by US Environmental Protection Agency (EPA)[14]. The approval by EPA was mainly due to a considerable factor that, no organism shall develop resistance against copper as they often do with antibiotics. This typical bactericidal effect of metal nanoparticles was mainly attributed due to their size, shape and high surface to volume ratio. This in turn makes the particles to interact closely with microbial internal membranes leading to damage, breakdown, lysis and cell death.

Based on these approaches, in the present research four different ratios of herbal and metal composite mixtures were prepared in nano sized particles. The dressing materials like bamboo and organic cotton were selected and surface coated with the prepared nanocomposite under a sterile condition with the aim of determining its antimicrobial activity and using it as a novel material for treating any types of wound infections. The study was performed with the following objectives:

- To optimize and synthesize herbal-copper oxide nanocomposite
- To study the coatings of nanocomposite for effective antimicrobial dressings and
- To determine the antimicrobial activity of prepared novel composite coated dressing materials against seven different strains of coagulase positive *Staphylococcus aureus*

MATERIALS AND METHODS

In the present research, herbal and metal composite synthesis, coating methodology and antibacterial activity of the dressing materials were carried out in PG and Research Department of Microbiology, GRD College of Science and Commerce, Coimbatore, India, from July, 2015 to December, 2015. Scanning electron microscopic analysis of coated dressing materials was performed at Indutech, PSG Centre of Excellence, Neelambur, Coimbatore, India. Medicinal herb, *Smilex china* (Parangi chakkai) was collected and authenticated from Tamil Nadu Agricultural University, Coimbatore, India. About 100% bamboo, 100% organic cotton as dressing materials was procured commercially from the local manufacturers, Coimbatore, India.

Solvent extraction of collected herbs[15]: Extraction was carried out by dissolving 6 g of the selected herbal powder Parangi chakkai in 100 mL 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[16]: Nanoparticles were synthesized by using 50 mL of herbal methanolic extract Parangi chakkai (*Smilex china*) Initially 125 mL of sodium alginate (base solution) (3.35 mg mL^{-1}) was prepared, followed by 75 mL of calcium chloride (3 mg mL^{-1}) was prepared. The calcium chloride ($CaCl_2$) solution was added a drop wise into sodium alginate solution with constant stirring at 1500 rpm for 30 min 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 min. 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 copper oxide nanoparticles[17]: Solution (1): About 6.9 g of copper sulphate pentahydarate was dissolved in 100 mL of distilled water. Solution (2): About 34.6 g of sodium potassium taratate and 12 g of sodium hydroxide was dissolved in 100 mL of distilled water.

Fifty milliliters of solution I and 50 mL of solution II was mixed together with vigorous stirring and 5 g of glucose (reducing agent) was added and then the mixture was stirred vigorously was 10 min and then keep in boiling water bath at 60°C for 10 min. Then, the obtained mixture is centrifuged and washed with distilled water twice and with ethanol twice and it was air dried and the powdered substance was used for further analysis.

Synthesis of antimicrobial herbal-metal nano composite: Herbal and metal nanocomposites were prepared in four different combinations (1:1, 1:2, 2:1 and 2:2). Single strength and double strength concentration of herbal and copper oxide metal nanoparticles was used to attain these ratio's. For 1:1 herbal metal nano composites, 100 mg of herbal nanoparticles were dispersed in 1 mL of sterile distilled water was added drop wise to 100 mg nano metal solution. Herbal solution was added at the rate of 1 mL min^{-1}. Similar procedure was carried out at controlled condition for the other ratios. All prepared antimicrobial composites at different ratio were further termed as HC_{NC} (herbal-copper oxide nano composites).

Preparation of antimicrobial wound dressing material: Coating on to bamboo and organic cotton materials using HC_{NC} by slurry-dipping technique[18]: Antimicrobial wound dressing materials coated with prepared HC_{NC} (herbal-copper oxide nano composites) were made using a standard slurry-dipping technique. The technique started with the preparation of stable slurry with specific amount of nanocomposite in the molten polyethylene glycol (PEG). Appropriate slurry temperature (37°C) was determined by an optimization process based on a trial and error approach to achieve optimum coating thickness, uniformity and stability of composite coating as well as adequate infiltration of drug

particles into coating structure. The PEG (2 g) with a predefined molecular weight was mixed with composite (0.5 g) in a glass vial. The mixture was heated at the range of 60.70°C in a water bath to obtain homogeneous slurry. The resulting slurry was homogenized in a magnetic stirrer for 5-10 min. Each dressing material was dip-coated twice with intermittent drying (suspension coating method). The dip-coating procedure was carried out in sterile glass beakers on a shaker (120 rpm) for 30 min with a drying period of about 15 min between the two coating procedures, followed by drying at room temperature. All coating steps were carried out under strict aseptic conditions. After coating procedure, the materials were stored at 4°C for upto 15 min. In order to increase antimicrobial drug loading and prevent excessive increase in material thickness, the coating process were repeated for replicates of each sample. Subsequently, in order to slow down the release rate of antimicrobial drug from PEG coating and mitigate the friction effect between material surfaces, second coating layer was formed using polyvinyl alcohol (PVA). The PVA was dissolved in DMSO to acquire a 10% (w/w) solution. PEG-coated materials were submerged into PVA solution three times for 1 min each. The coated materials were left to dry on a clean bench for 1 week at room temperature to remove residual DMSO, followed by determining the antimicrobial activity.

Antimicrobial activity of wound dressing materials coated with HC_{NC}-agar diffusion test: The antimicrobial activity of wound dressing materials (bamboo and cotton) coated with HC_{NC} was tested using a standard agar diffusion test. The materials coated with four different ratios (1:1, 1:2, 2:1 and 2:2) of HC_{NC} were tested against seven wound pathogenic strains of *Staphylococcus aureus*. Nutrient agar plates were prepared by pouring 15 mL of media into sterile Petri dishes. The plates were allowed to solidify for 5 min and 0.1% inoculum was swabbed uniformly and allowed to dry for 5 min. The wound dressing materials (coated with four different ratio, 1:1, 1:2, 2:1 and 2:2 of HC_{NC}) with the diameter of 2.0±0.1 cm was placed on the surface of medium and the plates were kept for incubation at 37°C for 24 h. At the end of incubation, the zone of inhibition formed around the material was measured in millimeters and recorded.

Examining the homogenous coatings on HC_{NC} biomaterials using topographic analysis-SEM: The surface coatings of the HC_{NC} (herbal-copper oxide nano composite) antimicrobial dress materials were observed using Scanning Electron Microscopy (SEM). The SEM evaluation was also used to know the homogenous coating of composites over the specimen. The topographic analysis of coated and uncoated test materials was prepared for SEM using a suitable accelerating voltage (10 kV), vacuum (below 5 Pa) and magnification (20,000X).

Hen's egg test on the chorioallantoic membrane-CAM test[19]: To study the allergic reactions or biocompatibility of HC_{NC} (herbal-copper oxide nano composite), the coated dressing materials (bamboo and cotton) were placed on the surface of chorio-allantoic membrane (CAM) of embryonated chick eggs. A standard HET-CAM protocol was followed to detect the inflammatory reactions. The HET-CAM (Hen's egg test-chorio allantoic membrane) method uses the vascular fetal membrane of chicken embryos. It is assumed that acute effects induced by a test substance and the small blood vessels and proteins of this soft tissue membrane are similar to effects induced by the same test substance in the skin of a treated rabbit. The membrane was evaluated for the development of irritant endpoints (vascular lysis, hemorrhage and coagulation) and qualitative assessments of the irritation potential of test substances are made.

Test substance preparation: All test substances, were evaluated undiluted. A 0.9% NaCl negative control should be included in each experiment in order to provide a baseline for the assay endpoints and to ensure that the assay conditions do not in appropriately result in an irritant response and 0.1 N NaOH was used as positive control.

Experimental design-treatment groups: About 4 eggs were selected, 2 eggs for test samples (bamboo and cotton coated with HC_{NC} separately) and 2 eggs for negative and positive control samples respectively. To the extent possible, eggs from the same hen should be randomized among treatment groups. Sample number and their respective designations were tabulated in Table 1.

CAM preparation: Selected fresh (not older than 7 days), clean, fertile 50-60 g White Leghorn chicken eggs. Candled the eggs and discarded any eggs that were nonviable or defective. Excessively misshapen eggs or eggs with cracked or thin shells were not used. Shaking, unnecessary tilting, knocking and all other mechanical irritation of the eggs should be avoided

Table 1: Sample numbers and its designation

Sample No.	Designation
1	Negative control (0.9% NaCl)
2	Positive control (0.1 N NaOH)
3	Bamboo coated with HC_{NC}
4	Cotton coated with HC_{NC}

Table 2: Relationship of scores with category of irritation[19]

Scores on HET-CAM	Category of irritation
0-0.9	No irritation
1-4.9	Weak or slight irritation
5-8.9	Moderate irritation
9-21	Strong or severe irritation

when preparing. Eggs were placed in an incubator with manual rotating (every 5 h eggs were rotated and placed). Eggs were incubated at 38.3±0.2°C in a still-air incubator or at 37.8±0.3°C in a forced-air incubator. Eggs were removed from the incubator on day 9 and used for the assay. Shells over the air sac layer in the eggs were marked as window of 1 inch^2. The window was made on the marked shell layer by cutting with a rotating dentist saw blade and then pared it off. Care should be taken when removing the eggshell to ensure that the inner membrane (CAM) was not injured.

Treatment of eggs with test substances: The test samples (bamboo and cotton coated with HC_{NC} separately) and another set of filter paper discs moistened with 0.9% NaCl and 0.1 N NaOH solution separately were kept ready for analysis. All the test samples were placed on the surface of the growing Chorio allantoic membrane of chick eggs and incubated for maximum of 5 min to observe the irritation endpoints.

Observations: The reaction on the CAM over a period of 5 min was observed manually using a stereo-zoom microscope. The time for the appearance of each of the noted endpoints was monitored and recorded, in seconds. Endpoints that should be observed were:

- Hemorrhage (bleeding from the vessels)
- Vascular lysis (blood vessel disintegration)
- Coagulation (intra and extra-vascular protein denaturation)

Irritation Score (IS) calculation-IS [B] analysis method (NIH, 2006): The time taken for the development of each endpoints, hyperemia, hemorrhage and coagulation was substituted in the standard formula as per IS [B] analysis method. The time values assigned/obtained to each endpoint were totaled to give an overall IS value for the test substance. An IS score could be calculated using the following general formula:

$$\left\{\left(\frac{(301-\text{Hemorrhage time})}{300}\right)\times 5\right\}+\left\{\left(\frac{(301-\text{Lysis time})}{300}\right)\times 7\right\}+\left\{\left(\frac{(301-\text{Coagulation time})}{300}\right)\times 9\right\}$$

Where:

Hemorrhage time = Time (sec) of the first appearance of blood hemorrhages

Lysis time = Time (sec) of the first appearance of vessel lysis

Coagulation time = Time (sec) of first appearance of protein coagulation

In Table 2 the final IS value ranged from 0 (for test substances that do not induce development of any of the observed endpoints) to 21 (for test substances that induce development of all three endpoints within 5 min of application of the test substance) was presented. The relationship between scores and category of irritation was given in Table 2.

RESULTS AND DISCUSSION

In the current medical scenario, wide spread applications of different systemic and topical antibiotics among the hospitalized and non-hospitalized patients increased the numbers of resistant bacterial strains. Therefore, potential use of antimicrobial wound dressing materials significantly loaded with specific antimicrobial agents have significantly prevented and treated the dissemination of microbial infections; also involved in effective wound healing in the individuals[20].

It was noted that wound tissue sites often get dried because of using topical antiseptic creams. These dried wound tissues will get delayed in wound healing[21]. Among the different wound dressing materials, moist wound healing strategies have found more significant in reducing the dissemination of infections. Moist wound healing materials keeps the exposed wound tissue at optimum hydration, so that the tissues would be found physiologically moist[22]. These moist wound healing materials have found to propose many advantages in treating wounds along with preventing scab formation, reducing the hydroxyl ion concentration at the wound tissue site, preventing the bacterial colonization from outside the wound to the inner wound tissues.

Saginur *et al.*[23] reported that the accepted clinical practice to treat bacterial wound infection was the use of combination therapy. This method of treating infection includes, blending two or more antimicrobial agents of different pharmaceutical groups. At lower concentrations, these 2 antimicrobial agents will achieve a broader spectrum of activity. This would also facilitate in resulting more effective clinical therapy and significantly decreasing bacterial resistance. One such approach was carried out in the present research by using a metal and herbal nanocomposite (HC_{NC}) containing copper oxide nanoparticles and *Smilax china*. Abo-Shosha *et al.*[24] designed the fabric to make multilayer, in which the inner layer was designed in such a way

Table 3: Antimicrobial activity of wound dressing materials (Bamboo) coated with HC_{NC}-agar diffusion test

			Zone of inhibition (mm) *Staphylococcus aureus*						
Nanocomposite	Ratio	Sample	1	2	3	4	5	6	7
Smilax china CuO	1:1		49	52	40	49	44	45	45
Nanocomposites	1:2	100%	40	42	42	44	52	51	50
(HC_{NC})	2:1	Bamboo	45	41	45	40	48	45	52
	2:2		41	40	41	43	44	47	45

HC_{NC}: Herbal (*Smilax china*) and copper oxide nanocomposite prepared in four different ratio after treating onto bamboo materials exhibits good antimicrobial activity against all test strains

Table 4: Antimicrobial activity of wound dressing materials (Cotton) coated with HC_{NC}-agar diffusion test

			Zone of inhibition (mm) *Staphylococcus aureus*						
Nanocomposite	Ratio	Sample	1	2	3	4	5	6	7
Smilax china CuO	1:1		44	49	40	48	44	46	39
Nanocomposites	1:2	100%	38	40	44	45	51	49	50
(HC_{NC})	2:1	Cotton	46	40	45	42	49	50	51
	2:2		43	46	44	38	45	43	44

HC_{NC}: Herbal (*Smilax china*) and copper oxide nanocomposite prepared in four different ratio after treating onto cotton materials exhibits good antimicrobial activity against all test strains

to have contact with skin that manifests high ability to absorb exudates and gelling behavior. The outer layer should also showed reasonable swelling to absorb any leaking exudates, without the necessity to manifest gelling. With the similar expectations, in the present study bamboo and cotton wound dressing materials were coated with metal and herbal nanocomposite (HC_{NC}) to resist local and invading infections that is to prevent the adherence of bacterial pathogens in both inner and outer layers. In the subsequent sections the composite coated materials were characterized by investigating their topography, antibacterial activity and biocompatibility.

Nanoparticles are special and interesting because their chemical and physical properties are different from their macro counterparts. Nanoparticles have unique properties due to their small size. The emergence of nanoscience and nanotechnology in the last decade presents opportunities for exploring the bactericidal effect of metal nanoparticles. 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. Alam *et al*.[25] suggested that the plant extract certainly posses some chemical constituents with antimicrobial properties. Based on this proposed mode of action of nanoparticles on the bacterial membrane, in the present study a novel nanocomposite containing herbal and metal formulation was developed. The composites were tested for its antimicrobial efficacy against medical significant coagulase positive *Staphylococcus aureus*. Bisi-Johnson *et al*.[26] 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 dressing material coated with the nanocomposite was aimed to prevent such critical cases with diabetic foot ulcers.

Antimicrobial activity of wound dressing materials coated with HC_{NC}-agar diffusion test: Antimicrobial activity of herbal-metal nano composite (HC_{NC}) synthesized in different ratios was expressed as Inhibition Clear Zone (ICZ) against all the test strains of *Staphylococcus aureus.* Different significant factors like herbal drug and metal oxides in the composite highly influenced the inhibition of the growth of bacteria around four different ratios of HC_{NC} coated wound dressing materials (Bamboo). From Table 3, 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 40-52 mm for all the ratios against all the test strains. Maximum ICZ of 52 mm was observed for three different ratios, 1:1, 1:2 and 2:1 against test strains 2, 5 and 7, respectively and the lowest of 40 mm for all the ratios against test strains 3, 1, 4 and 2. Similar observations in antimicrobial activity were noted for the 2nd wound dressing material (cotton) coated with four different ratios of HC_{NC}. Significant ICZ was observed for the coated materials against all the seven strains of *Staphylococcus aureus*. In Table 4, it was evident that no significant changes in the

Table 5: Comparative evaluation of irritation scores for test materials, negative control and positive control by HET-CAM test

	Endpoint development			
Materials on CAM	Haemorrhage	Hyperemia	Coagulation	Irritation score[1]
Sample-1 (Negative control)	0	0	0	0
Sample-2 (Positive control)	5.7	6.5	6.9	19.1[2]
Sample-3 Bamboo coated with HC_{NC}	0	0	0	0
Sample-4 Cotton coated with HC_{NC}	0	0	0	0

[1]Irritation score calculated as described by IS [B] analysis, [2]Irritation category-severe irritation

Inhibition Clear Zone (ICZ) were observed for any of the composite ratios specifically. The ICZ range from 38-51 mm against all the test strains when tested for all the sample materials coated with four different ratios of HC_{NC}. Maximum ICZ of 51 mm was observed for the ratio, 2:1 against test strains-7, respectively and the lowest of being 38 mm for the ratios, 1:2 and 2:2 against test strains 1 and 4, respectively.

Similar pattern of difference in antibacterial activity was observed in our previous study[27]. The antibacterial activity of carboxy methylated-antimicrobial dressing material (CM-AMD) was reported against the test organisms *Staphylococcus aureus* and *Peptostreptococcus* sp. using similar ICZ method. Different significant factors like antibacterial drug, carboxyl and Ca contents highly influenced the inhibition of the growth of bacteria around CM-AMD samples. During the study, it is experienced that the ICZ was increased after increasing the drug concentration and carboxyl content (85, 173 and 246 mmol 100 g). Also it was observed that ICZ was decreased by increasing Ca content (20 and 30% degree of neutralization). The reason was reported that, increasing the extent of cross linking of the material structure by the divalent Ca cations (20 and 30%) would restrict the diffusion of antibacterial drugs from CM-AMD fabrics.

Antimicrobial efficacy of the HC_{NC} coated wound dressing materials has a characteristic feature of synergism. It was clearly understood from their mode of action on bacterial cell components. Surfaces of copper nanoparticles affect/interact directly with the bacterial outer membrane (peptidoglycan), causing the membrane to rupture and killing bacteria. Kim *et al.*[28] emphasized that the bactericidal effects observed in this study might have been influenced by the release of Cu^{2+} ions. Copper ions released by the nanoparticles may attach to the negatively charged bacterial cell wall and rupture it, thereby leading to protein denaturation and cell death. Copper ions inside the bacterial cells may bind to deoxyribonucleic acid molecules (DNA) and become involved in cross-linking within and between the nucleic acid strands, resulting in the disorganized helical structure[29]. In the previous study, 2 drug combinations (ofloxacin-fluoroquinolone and ornidazole-nitroimidazole) were selected mainly based on their mode of action on DNA of prokaryotic organisms. Like the mode of action of copper 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[30].

Hen's egg test on the chorioallantoic membrane histological evaluation of CAM: The test samples (bamboo and cotton wound dressing material coated with HC_{NC}) do not develop any irritant end points, which revealed that the samples were biocompatible in nature. The obtained results were thus compared with negative control samples, which also showed no irritant endpoints. Whereas, the positive control samples showed all the three irritant endpoints. The positive control Irritation Score (IS) was calculated using the time taken for the development of identified endpoints based on the standard formula. The mean value of time for the development of hemorrhage, hyperemia and coagulation were identified as 5.7, 6.5 and 6.9, respectively (Table 5).

Histological evaluation of test materials implanted CAM was observed under a bright-field microscope after staining with haematoxylin and eosin. Strong blue and hue colors of inflammatory cells due to fibrous deposition on positive control CAM samples (0.1 N NaOH) were observed. The active ingredient, hematein complexed with aluminium potassium sulphate in haematoxylin produced the strong blue color and eosin produced the shades of hue colors. In Fig. 1, the three endpoints like haemorrhage hyperemia and coagulation which were observed by naked eye was presented. Negative control (0.9% NaCl) CAM sample showed well developed blood vessels and nucleated epithelial cells (Fig. 2). Histological examination of the test material implanted CAM samples were compared with the interpretations of positive and negative control samples. The CAM implanted with test materials, bamboo and cotton impregnated with HC_{NC} samples showed no tissue reactions

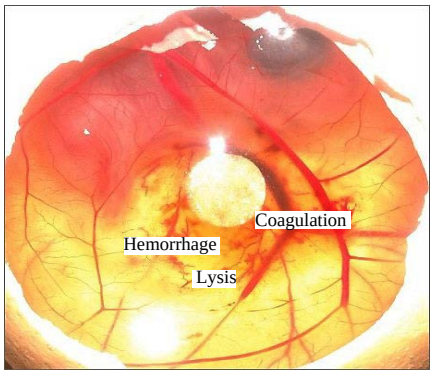

Fig. 1: Positive control CAM sample. All three irritation types like lysis of bleed vessels, haemorrhage and coagulation on the chorioallantoic membrane of chick embryo was observed for the positive control (NaOH) coated sample

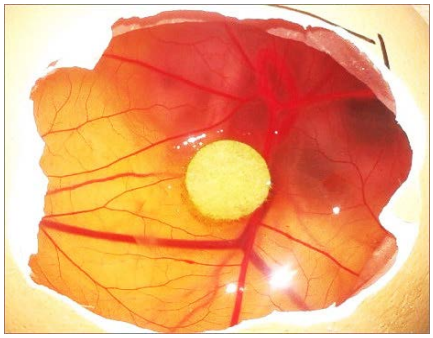

Fig. 2: Negative control CAM sample. No Irritation types on the membrane of chick embryo was observed for the sample coated with negative control sodium chloride

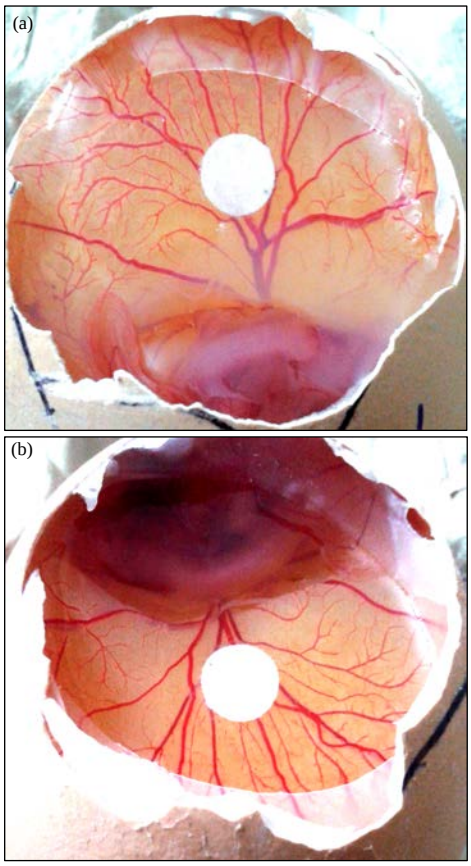

Fig. 3(a-b): (a) Bamboo coated HC_{NC} and (b) Cotton coated HC_{NC}. No Irritant endpoints were observed for both the materials coated with HC_{NC} thus indicating the biocompatible properties of the nanocomposite

or necrosis after comparing with the positive and negative control CAM samples (Fig. 3a, b). Even the possible mild tissue reactions like obvious edema and fibrous deposition with degenerative changes of epithelial cells was not observed for the test material. Instead clear well developed nucleated epithelial cells with blood veins were observed indicating good biocompatible properties of HC_{NC} materials. The CAM model is a true *in vivo* system that can be used as an intermediate step between a cell culture and a more complex mammalian model. The CAM can be used for the evaluation of both acute and chronic inflammatory responses to biomaterials[31]. In addition, the CAM model used in the study presented the ability to continuously visualize the implant site without having to sacrifice the test animal.

Examining the homogenous coatings on biomaterials using topographic analysis-SEM: Scanning electron microscope was used to identify morphological structure of the antimicrobial dressing materials under investigation. SEM evaluation was also used to know the uniformity of coating of finishing drugs or chemicals over the material. The antimicrobial dressing materials (bamboo and organic cotton) were observed visually and the topography of these samples was analysed using SEM with suitable accelerating voltage (10 kV), vacuum (below 5 Pa) and magnification (3500X).

Scanning electron microscope was used to examine if the treatment finishes (coatings) were applied successfully on the test materials. The SEM was also used to project an image of each material to examine visually any differences in the material surface, across the test materials. If the coatings were evenly applied on the material surface through treatment, the surface of materials was expected to be smoother and more uniform than the uncoated material surface. Usually the surfaces of materials coated with nanocomposites will look smooth and uniform. The nanoparticles will be well dispersed

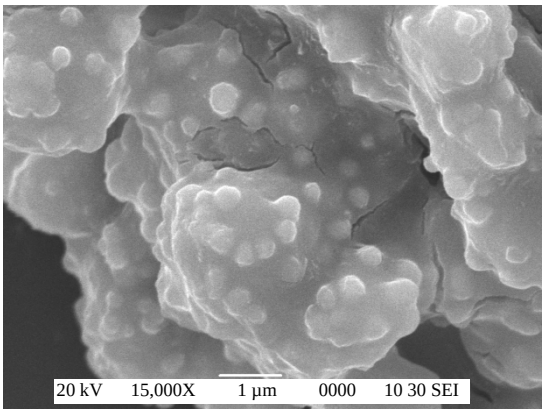

Fig. 4: SEM micrograph of HC_{NC} coated onto bamboo materials (15,000X magnifications). Irregular aggregated shape of HC_{NC} at 15,000 magnifications besides its diameter ranges from 1 µm

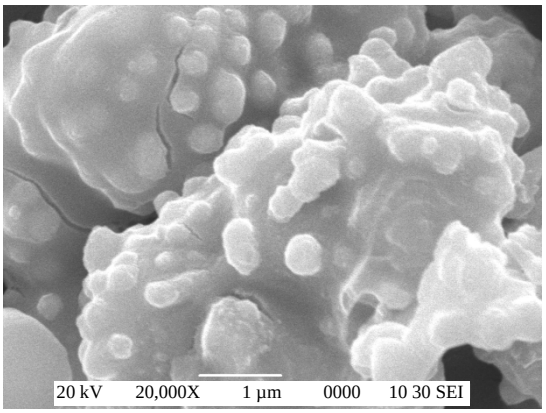

Fig. 5: SEM micrograph of HC_{NC} coated onto cotton materials (20,000X magnifications). Irregular aggregated shape of HC_{NC} at 20,000 magnifications besides its diameter ranges from 1 µm

on the surface of coated materials with homogeneous distribution in the coating layer, thus making the coated materials to have uniform antimicrobial property.

But interestingly, when the morphology of prepared HC_{NC} was studied by Scanning electron microscope at 15,000 and 20,000 magnifications (Fig. 4 and 5), aggregates in various forms with the average diameter of the nanoparticle ranging from 1 µm was technically observed. Aggregation refers to the collection of nanoparticles that are held together by van der Waals and electrostatic interaction. Under ambient conditions, CuO nanoparticles can form aggregates or agglomerates in various forms. Jayasree *et al.*[32] and Mustafa *et al.*[33] obtained aggregates of CuO nanoparticles when observed under scanning electron microscopy. Thus from this topographical investigation, we revealed that the irregular aggregated shape of CuO nanoparticles at 10000 magnifications besides its diameter ranges from 1-5 µm and 0.2-1 µm, respectively. The visual examination of the SEM indicated that the antimicrobial treatments were applied successfully to each test material.

Similar observations were obtained by Chellamani *et al.*[34] for the nano-scaled silver and fluorocarbon particles on cotton and polyester/cotton samples. They observed that the nanoparticles were well dispersed on the fibre surface in both the cases, thus making the coated materials to have uniform antimicrobial and liquid repellent property. Parthasarathi and Thilagavathi[35] analyzed the presence and sizes of antimicrobial nano particles of zinc on the material surface using SEM. In their report they suggested that the nano particles were well dispersed on the fiber surface, although some aggregated nanoparticles were still visible. Another significant characteristic that would influence the coating of particles on the material surface was determining the particle size. The particle size plays a major role in determining their adhesion to the fibre molecules; generally agglomeration of large particles will get easily removed from the fibre surface, while the small particles will penetrate deeper and adhere strongly into fabric matrix[36]. In the present study, nano sized particles were coated to fix firmly on the fibre assembly of the organic cotton and bamboo materials to enhance their antimicrobial efficacy and durability.

CONCLUSION

Wound healing is considered as one of the significant pharmacological applications according to the recent survey. Many wound dressing materials were demonstrated against pathogenic strains isolated from burn wounds and diabetic foot ulcers. Even though many metal nanoparticles coated wound dressing materials were commercially available, the present study revealed the pharmacological application of a novel metal and herbal nanocomposite (HC_{NC}) in two different dressing materials, bamboo and cotton. Antibacterial activity of the HC_{NC} coated bamboo and cotton showed remarkably good inhibitory clear zones against *Staphylococcus aureus*. The synthesized HC_{NC} are composed of irregular shapes and aggregates of copper oxide nanoparticles with the average diameter of 1 µm, which provide a large surface area for the adsorption is determined by SEM results. The biocompatible properties of the coated materials also proved to be much promising when tested using HET-CAM assay. As a future perspective, the HC_{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. The wound healing properties of the composite containing HSEs can be studied using standard assays like scratch wound healing assay and boyden chamber migration assay without sacrificing any animals. Thus the present investigation was proved to be a preliminary experiment for the development of a novel pharmacological product in future.

REFERENCES

1. White, R.J., 2002. The use of topical antimicrobials in wound bioburden control. Br. J. Community Nursing, 7: 20-26.
2. Fletcher, J., 2006. Antimicrobial dressings in wound care. Nurse Prescribing, 4: 320-326.
3. Morison, M. and C. Moffatt, 1999. Leg Ulcers. In: A Colour Guide to the Nursing Management of Chronic Wounds, Morison, M., C. Moffatt, J. Bridel-Nixon and S. Bale (Eds.). Mosby Inc., London.
4. Flores, A. and A. Kingsley, 2007. Topical antimicrobial dressings: An overview. Wound Essentials, 2: 182-185.
5. Kingsley, A., 2001. A proactive approach to wound infection. Nursing Standard, 15: 50-58.
6. Frost, M.R., S.W. Jackson and P.J. Stevens, 1980. Adsorption of bacteria onto activated charcoal cloth: An effect of potential importance in the treatment of infected wounds. Microbios Lett., 13: 135-140.
7. Adams, A.P., E.M. Santschi and M.A. Mellencamp, 1999. Antibacterial properties of a silver chloride-coated nylon wound dressing. Vet. Surg., 28: 219-225.
8. Stemberger, A., H. Grimm, F. Bader, H.D. Rahn and R. Ascherl, 1997. Local treatment of bone and soft tissue infections with the collagen-gentamicin sponge. Eur. J. Surg. Suppl., 578: 17-26.
9. Summerhays, G.E., 2000. Treatment of traumatically induced synovial sepsis in horses with gentamicin-impregnated collagen sponges. Vet. Rec., 147: 184-188.
10. Colella, G., A. Vicidomini, N. Cirillo, G.M. Gaeta and S. D'Amato, 2010. Aminoacid-enriched sodium hyaluronate enhances keratinocyte scattering, chemotaxis and wound healing through integrin b1-dependent mechanisms. J. Stomatol. Invest., 4: 21-29.
11. Ahmad, I. and A.Z. Beg, 2001. Antimicrobial and phytochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. J. Ethnopharmacol., 74: 113-123.
12. Na, H.K., E.H. Kim, M.A. Choi, J.M. Park, D.H. Kim and Y.J. Surh, 2012. Diallyl trisulfide induces apoptosis in human breast cancer cells through ROS-mediated activation of JNK and AP-1. Biochem. Pharmacol., 84: 1241-1250.
13. Theivasanthi, T. and M. Alagar, 2010. X-ray diffraction studies of copper nanopowder. Archiv. Phys. Res., 1: 112-117.
14. ECHA., 2008. Voluntary risk assessment copper and copper compounds. Final Report Submitted to the European Chemicals Agency (Helsinki, Finland) by the European Copper Institute, Brussels, Belgium.
15. Tiwari, P., B. Kumar, M. Kaur, G. Kaur and H. Kaur, 2011. Phytochemical screening and extraction: A review. Internationale Pharmaceutica Sciencia, 1: 98-106.
16. Moradhaseli, S., A. Mirakabadi, A. Sarzaeem, M. Kamalzadeh and R.H. Hosseini, 2013. Cytotoxicity of ICD-85 NPs on human cervical carcinoma HeLa cells through caspase-8 mediated pathway. Iran. J. Pharm. Res., 12: 155-163.
17. Kooti, M. and L. Matouri, 2010. Fabrication of nanosized cuprous oxide using Fehling's solution. Trans. F: Nanotechnol., 17: 73-78.
18. Boccaccini, A.R., I. Notingher, V. Maquet and R. Jerome, 2003. Bioresorbable and bioactive composite materials based on polylactide foams filled with and coated by Bioglass® particles for tissue engineering applications. J. Mater. Sci.: Mater. Med., 14: 443-450.
19. Cazedey, E.C.L., F.C. Carvalho, F.A.M. Fiorentino, M.P.D. Gremiao and H.R.N. Salgado, 2009. Corrositex®, BCOP and HET-CAM as alternative methods to animal experimentation. Braz. J. Pharmaceut. Sci., 45: 759-766.
20. White, R.J., R. Cooper and A. Kingsley, 2001. Wound colonization and infection: The role of topical antimicrobials. Br. J. Nursing, 10: 563-578.
21. Venkatrajah, B., V.V. Malathy, B. Elayarajah, R. Rajendran and R. Rammohan, 2013. Synthesis of carboxymethyl chitosan and coating on wound dressing gauze for wound healing. Pak. J. Biol. Sci., 16: 1438-1448.
22. Ovington, G.L., 2007. Advances in wound dressings. Clin. Dermatol., 25: 33-38.
23. Saginur, R., M. StDenis, W. Ferris, S.D. Aaron, F. Chan, C. Lee and K. Ramotar, 2006. Multiple combination bactericidal testing of staphylococcal biofilms from implant-associated infections. Antimicrob. Agents Chemother., 50: 55-61.
24. Abo-Shosha, M.H., H.M. Fahmy, F.H. Hassan, A.M. Ashour and A.A. Khalil, 2009. Tetracycline hydrate and gentamicine sulfate containing carboxymethylated cotton fabric suitable for moist wound healing dressings: Properties and evaluation. J. Ind. Text., 38: 341-362.
25. Alam, K.D., M.S. Ali, S. Parvin, S. Mahjabeen, M.A. Akbar and R. Ahamed, 2009. *In vitro* antimicrobial activities of different fractions of *Swertia chirata* ethanolic extract. Pak. J. Biol. Sci., 12: 1334-1337.
26. Bisi-Johnson, M.A., D.O. Kolawole and A.O. Shittu, 2005. Epidemiological analysis of clinical isolates of *Staphylococcus aureus* in Ile-Ife, Nigeria. Pak. J. Biol. Sci., 8: 1016-1020.
27. Balasubramanian, E., V. Balasubramanian, G. Babu, S. Devika and R. Rajendran, 2013. Moist wound dressing fabrications: Carboxymethylation of antibacterial cotton gauze. J. Eng. Fibers Fabrics, 8: 78-87.

28. Kim, K., C.D. Harvell, P.D. Kim, G.W. Smith and S.M. Merkel, 2000. Fungal disease resistance of Caribbean sea fan corals (*Gorgonia* spp.). Mar. Biol., 136: 259-267.
29. Haque, M.E., M.Z. Rahman, M.M. Pervin, M.H. Kabir and M.S. Imran, 2005. Biological screening of some ferrocene derivative metal complexes. Pak. J. Biol. Sci., 8: 1746-1750.
30. Drlica, K. and X. Zhao, 1997. DNA gyrase, topoisomerase IV and the 4-quinolones. Microbiol. Mol. Biol. Rev., 61: 377-392.
31. Valdes, T.I., D. Kreutzer and F. Moussy, 2002. The chick chorioallantoic membrane as a novel *in vivo* model for the testing of biomaterials. J. Biomed. Mater. Res., 62: 273-282.
32. Jayasree, K.V., K. Neelakanndeswari, B. Elayarajah and R. Rajesh, 2015. Synthesis and characterization of copper oxide nanoparticles against dental implant associated infections. Int. J. Applied Eng. Res., 10: 2895-2908.
33. Mustafa, G., H. Tahir, M. Sultan and N. Akhtar, 2013. Synthesis and characterization of cupric oxide (CuO) nanoparticles and their application for the removal of dyes. Afr. J. Biotechnol., 12: 6650-6660.
34. Chellamani, K.P., D. Veerasubramanian and G. Panneerselvam, 2010. Breathability of woven surgical gowns treated with nano finishes. South India Textile Research Association, India.
35. Parthasarathi, V. and G. Thilagavathi, 2011. Synthesis and characterization of zinc oxide nanopartilce and its application on fabrics for microbe resistant defence clothing. Int. J. Pharm. Pharmaceut. Sci., 3: 392-398.
36. Martinez-Castanon, G.A., N. Nino-Martinez, F. Martinez-Gutierrez, J.R. Martinez-Mendoza and F. Ruiz, 2008. Synthesis and antibacterial activity of silver nanoparticles with different sizes. J. Nanoparticle Res., 10: 1343-1348.