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Original Article

Orthopedic application of spikenard herbal rhizome decorated microstructured polymer biocomposites and their *in vitro* cytotoxicity

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ABSTRACT

The present study explores the synthesis of highly potential polymer biocomposite from *Nardostachys jatamansi* rhizome extract. The polymer biocomposites were synthesized from methyl methacrylate by free radical polymerization. ATR-IR enunciate the functional groups attributed at 956 cm^{-1} (aromatic), a peak appeared at 1685 cm^{-1} (–C=O), 1186 cm^{-1} (–O–CH_3), 1149 cm^{-1} (–C–O–C) framework and 1279 cm^{-1} (–C–O), which are good agreement for the formation composites. The quantitative evaluations of antimicrobial studies were analyzed by serial dilution method and also improved activity in orthopedic infection pathogens. Cytocompatibility was analyzed by keratinocyte cell lines and it may be used for various biomedical applications.

1. Introduction

In recent years microbes have developed resistance and their pathogenic ability was increased due to the repeated use of common commercial drugs by the people for treatment of various diseases. Many research articles have reported that the herbal medicine does not cause any side effect and they are important alternatives to synthetic medicines.[1] *Nardostachys jatamansi* is one of the most popular aromatic medicinal plant and an endangered, therapeutic agent belongs to the family of valerianaceae, locally called spikenard. It is a very rare plant found in Kolli hills and Himalayas in India. *N. jatamansi* was used in traditional medicines and cosmetic products for centuries.[2,14,15] More than 25 active potential compounds have been isolated from the rhizomes of plant. The major alkaloids namely jadamonson, nardostachone, coumarins and neoligns are present in this plant.[3] *N. jatamansi* rhizomes are used to treat skin diseases, mental illness, hypertension, epilepsy, cholera, hyperlipidemia and heart diseases.[4,5] Zahida et al.,[6] stated that the essential oil of *N. jatamansi* has potential antimicrobial activity against both gram positive and gram negative bacteria.

Pandey et al.,[7] reported that the phenolic acid components are powerful antioxidants and they have several biological activities against bacteria, virus, cancer cells and inflammation.[8] Krishna Rao et al.,[9] developed a very transparent polymer biocomposite film using this plant rhizome and tested against various fungal pathogens. In these days, we are all depending upon bio-based synthetic materials in all fields especially in medical field.[10,11] The polymethyl methacrylate (PMMA) has high mechanical strength and good stable quality and it can be used as a transparent engineering material such as contact lens, tooth resin and bone cement.[12] The smooth surface having PMMA microspheres are ideal fillers for soft tissue in cosmetics and reconstructive application.[16]

Cataract surgery has great attention in order to develop a new posterior capsule opacification after intraocular lenses implantation for cataract lens.[17] During cataract surgery, human lens epithelial cells (HLECs) were severely damage. In order to resolve this problem, Wang et al.,[18] synthesized a novel poly(hedral oligomeric silsesquioxane-co-methyl methacrylate) copolymer using free radical polymerization technique to promoting the *in vitro* cytotoxicity of HLECs cells and silver nanoparticles embedded PMMA composites could be used in the field of anti orthopedic infection against *Acinetobacter baumannii* and methicillin resistant *Staphylococcus aureus*. This antimicrobial implant material could act as broad spectrum and long intermediate term antimicrobial effect.[19–21]

Moreover, PMMA has more biocompatible and it can also enhance better anti-microbial activity.[13] With this background the present work was carried out to synthesized Jatamansi-based polymer biocomposite using specific monomer of methyl methacrylate by free radical polymerization technique and the

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biocomposites was tested against various orthopedic bacterial and fungal pathogens. More particularly, cytotoxicity was done using human keratinocytes cells.

2. Materials and methods

N. jatamansi plant rhizomes were collected from Kolli hills, Namakkal district, Tamil Nadu, India. The rhizomes were separated from plant by cutting washed, shade dried, pulverized into powder form and powder materials were stored in air-tight bottles. The monomer of methyl methacrylate (MMA) with formula of $C_5H_8O_2$-99% contained $\leq$30 ppm, monomethyl ether hydroquinone as an inhibitor and radical initiator of benzyl peroxide was purchased from Sigma–Aldrich, USA. Keratinocyte cell lines from adult human skin (HaCaT cells) were procured from National Center for Cell Science (NCCS), Pune, India. Dulbecco's modified Eagle's medium (DMEM), trypsin-EDTA, fetal bovine serum (FBS), 3-(4,5-dimethyl thiazol-2-yl)-2,5-(diphenyl tetrazolium bromide) (MTT), antibiotic-antimycotic solution, and other chemicals were purchased from Sigma–Aldrich, USA. Chloroform, methanol (CH_3-OH), Nutrient agar and broth, Agar Agar type-I, sterile disk, potato dextrose agar and broth were procured from Hi-media for microbiological examination. The bacterial and fungal culture strains were collected from MTCC, Chandigarh, India.

The solution state ^{13}C NMR spectroscopic analysis was recorded on a Bruker Advance III 400 NMR spectrometer (400 MHz, Germany) and the spectra were recorded in $CDCl_3$ solvent. The new JASCO model V-530 UV-vis spectrophotometer (USA) was recorded in chloroform. FT-IR spectra of the polymer composite films were taken using ABB MB3000 Fourier transform infrared spectrometer in ATR mode, 40 scans with resolution of 12 cm^{-1}. Optical microscopy was performed using Olympus BX50 optical polarizing microscope and photograph was taken in an Olympus C7070 using digital camera. The topographical analysis determined by using atomic force microscopy (AFM), conducted in non-contact mode, NT-MDT, NTEGRA Prima, Netherlands. The thermo lab systems multiskan ascent photometer for 96 well plates was used for cell assessment and cell morphological analysis was determined by fluorescence microscopy (DMI-IL LED, Leica, Germany).

2.1. Sample preparation for atomic force microscopy

The rhizome extract based polymer composites (1 mg) was dissolved in chloroform (1 mL) using sonicator for about 2 min. 1 cm^2 glass substrates were prepared by diamond cutter and clean the substrate using methanol and acetone in order to avoid dust particles. We preferred non-contact mode, because this mode has advantage that cantilever tip never make contact with sample and it cannot disturb or destroy the sample surface. Most probably, many biological samples could be used in non contact mode in AFM.[24] During sample preparation, surface charges surface energy, flatness of substrate and hydrophobicity are crucial role.[25] However, 3 μL of compound was taken by using micropipette, transferred onto clean glass substrate and dried at 40 °C under vacuum oven. Operational condition for AFM experiment, set point, scan size and scan speed was adjusted to take better resolution topographical images. The dried glass substrate was used for both AFM and optical microscopy to analyze their morphology.

2.2. Preparation of N. jatamansi rhizome extract

The extraction method is very essential in medicinal plants because of its desired chemical components from plant materials. Here, we describe the basic and simple operational extraction steps include prewashing, drying, grinding to get homogenous materials. First 50 g of *N. jatamansi* rhizome was taken in a 250 mL of beaker and wash several times with normal tap water to remove soil and dust particles. Again wash with double distilled water about 15 min then completely dried at air atmosphere and kept it in closed room for shadow dry about two weeks. The compound was ground into fine powder and 40 g of bioactive compound was extracted from rhizome by using polar solvent methanol by soxhlet extraction method in the ratio of 1:6 and kept it in shaker for three days at ambient temperature.[22,23] Finally, plant rhizome material was filtered using Whatman filter paper, kept it in vacuum oven for complete drying and stored at 37 °C.

2.3. Preparation of Jatamansi-based polymer composites

The monomer of methyl methacrylate (4 mL) and the radical initiator of benzoyl peroxide (0.05 g) were taken in a double neck round bottom flask. The initiator of benzyl peroxide was dissolved in 3 mL of distilled chloroform. The initiator was added drop by drop using glass syringe to induce the polymerization. Before that nitrogen gas was purged for 30 min and this whole reaction was maintained under nitrogen atmosphere with stirring condition for 3 h at 80 °C. The bioactive compound of *N. jatamansi* rhizome (3%) extract was dissolved in 3 mL of distilled chloroform and this extract was incorporated once again into the polymerization system for 30 min at ambient temperature. Finally, composite material was removed from oil bath and poured into glass petridish to cast as uniform thickness film.

2.4. Types of microbes and culture conditions

The polymer biocomposite was examined their therapeutic activities by using both gram negative and gram positive bacteria such as *Streptococcus pneumoniae*, *Streptococcus aureus*, *Enterobacter aerogenes*, *Salmonella paratyphi*, *Serratia marcescens*, *Escherichia coli*, *Staphylococcus aureus* and fungal pathogens namely *Malassezia pachydermatis*, *Aspergillus niger*, *Trichophyton rubrum*, *Candida tropicalis* and orthopedic infection causing pathogens *S. marcescens*, *E. coli*, *Staphylococcus aureus* and *C. tropicalis*. Here, the entire microbes were cultured overnight in nutrient agar medium (Hi-media). The prepared microbial inoculum colonies were transferred into 10 mL nutrient broth tube. The microbes containing tubes were shaken for aeration to promote their microbial growth and incubated at 37 °C for 24 h.[27]

2.5. Determination of minimum inhibitory concentration (MIC)

Human bacterial pathogens *S. pneumoniae*, *E. aerogenes*, *S. paratyphi* and *Streptococcus aureus* and pathogenic fungi such as *M. pachydermatis*, *T. rubrum*, *A. niger* and *C. tropicalis* were used for testing. In addition, orthopedic infection pathogen of *S. marcescens*, *E. coli*, *Staphylococcus aureus* were used for MBC. All the microbial cultures were maintained at 4 °C on nutrient agar and potato dextrose agar (PDA) respectively. The MIC of PMMA, *N. jatamansi* rhizome extract and biocomposite were determined by serial dilution method. The PMMA (200 mg), *N. jatamansi* rhizome extract (200 mg) and biocomposite materials (200 mg) were dissolved in chloroform and DMSO in concentration 200 mg/mL were mixed with nutrient broth (for bacteria) and potato dextrose broth (for fungi), then 100 μL of test inoculums were added into each tube (10^{-1} to 10^{-10} dilution). The final concentration of PMMA, rhizome extract and biocomposite ranged from 0.1 to 40 mg/mL and test tubes were incubated at 37 ± 2 °C for 24–48 h by downstream dilution. The MIC defined as the lowest concentration of antimicrobial that inhibited the growth of microorganism of the incubation was defined as turbidity nature, which clearly indicate the