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PREPARATION, OPTIMIZATION, CHARACTERIZATION AND ANTIMICROBIAL ACTIVITY OF CHITOSAN AND CALCIUM NANOPARTICLES LOADED WITH *STREPTOMYCES RIMOSUS* EXTRACTED COMPOUNDS AS DRUG DELIVERY SYSTEMS

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ABSTRACT

The present study aims to search for a nano-delivery system that enables access of antimicrobial compounds to the targeting site of the microbial pathogens. To achieve this objective, the antimicrobial compounds extracted from the local isolate *Streptomyces rimosus* were loaded on chitosan (CS) and calcium phosphate (CaP) nanoparticles (NPs) to facilitate drug delivery to some species of bacteria (*Escherichia coli* ATCC25922, *Staphylococcus aureus* ATCC25923 and *Bacillus cereus* ATCC6633) and the yeast *Candida albicans* ATCC10231. The best incubation period for the production of antifungal and antibacterial compounds, pH, temperature, carbon sources and nitrogen sources were around the third day, 7.0, 30°C, starch and potassium nitrate, respectively. The extracted compounds were identified using gas chromatography-mass spectrometry (GC-MS) analysis which revealed to the identification of nine antimicrobial organic acids. The prepared NPs were characterized using transmission electron microscopy (TEM) and Zeta potential analyser. Results showed those chitosan nanoparticles (CSNPs) were spherical shaped NPs with an average size ≈22 ± 2 nm. The CSNPs loaded with the antimicrobial molecule's diameter was ranged from 55 upto 100 nm. Calcium phosphates nanoparticles (CaPNPs) were also spherical shaped NPs with diameter range from 70 to 95 nm. Loading of antimicrobial compounds onto CaPNPs made them appeared in rod shape with width varying from 14 to 27 nm and length from 274 to 397 nm. The synthesized NPs showed negative values confirming their high dispersity and long-term stability. The tested strains were resistant to solo NPs; but the loaded NPs with the extracted antimicrobial compounds, particularly the CaPNPs, augmented the potency of the isolated antimicrobial compounds, with emergence of differential antimicrobial activity. The activity of the NPs-bound antimicrobial compounds was more evident against bacteria than fungi, Gram-positive bacteria than Gram-negative bacteria and against *B. cereus* than *S. aureus*.

Keywords: chitosan, calcium phosphate, nanoparticles, *Streptomyces rimosus*, optimization, antimicrobial

INTRODUCTION

Microbial diseases are increasing yearly which represents an enormous challenge to human health, especially the improvement of antibiotic resistant strains in pathogenic microbes that considered as an alarming issue worldwide **(Meade et al., 2021)**. It is well- established that the current medicinal regime delivers drugs to the site of action or inflammation with inescapable side effects **(Małgorzata et al., 2020)**. Nanotechnology provides an efficient mean of sustained drug delivery and release with avoidance of the draw backs of the current delivery systems. For proper manipulation, medicaments must have a size such that they can be injected without occluding needles and capillaries **(Hughes, 2005)**, and this task can be achieved by using either nano-liposomes, nano-gels, or micelles. By manipulating nanotechnology, drugs can be either loaded on the surface of nanoparticles (NPs) or encapsulated and carried within them to the drug destination. By this way, the effective drug dose can be lowered several orders of magnitude, which permits minimization of the drug side effects **(Campagnolo et al., 2013)**. Chitosan (CS) is a safe natural biopolymer synthesized by basic de-acetylation of N-acetyl glucosamine polymer (chitin) gotten from polymer, chitin, chitosan has drawn in critical consideration as an expected possibility for drug delivery because of its low poisonousness, biocompatibility and great biodegradability **(Babii et al., 2020)**. Cs and its derivatives, bulk, or NPs have attracted considerable attention owing to its antimicrobial activity **(Tsai and Su, 1999; Baka et al., 2020)**. The safe CS can interact with anions forming gels and complexes **(Agnihotri et al., 2004; Kim and Rajapakse, 2005)**. CS hydrophilic property can simply cross-interface with counter anions to manage the drugs release **(Hu et al., 2002)**. CS is a hydrophilic with mucoadhesion property **(Janes et al., 2001)**, permeation enhancing properties **(Dudhani & Kosaraju, 2010)** which ease the epithelial tight junctions opening **(Yamamoto et al., 2005)**.

Calcium phosphates (CaP) are the most important mineral component of human bone and teeth as well as the biological hard tissues in the form of carbonated hydroxyapatite, which afford stability, hardness, and proper function **(Dorozhkin and Epple, 2002; Epple and Baeuerlein, 2007)**.
Synthetic CaP showed high biodegradability and biocompatibility and it had wide use in bioimaging, nanotechnology, medicine and tissue culture **(Bakan et al., 2021)**. In addition, CaPs might equipped for separating into particles inside the cells and this property makes them fitting specialists for intracellular vehicle of the therapeutic genes and molecules **(Bakan, 2018)**.
Calcium phosphates nanoparticles (CaPNPs) were manipulated as successful adjuvant with DNA vaccines **(Joyappa et al., 2009)**. As the correspondence between the solubility of the carrier is inevitable and the antibiotic elution rate, CaPNPs is predictable to reveal slow-release performance **(Wu et al., 2020)**. Since CaPNPs has high chemical stability, it limits some of its conceivable applications. Thus, soluble CaP could be benefited once more rapidly biodegradation achieved **(Khalifehzadeh & Arami, 2020)**. **Goldschmidt et al. (2021)** stated the antimicrobial action of CaP against *S. aureus*. **Beigoli et al. (2021)** demonstrated the antibacterial action of CaPNPs against *S. mutans* and *E. Faecalis*. He described its mode of action involving microbial DNA damage and enzymes deactivation. This action differs attributed to the species of bacteria and its structure. CaPNPs could adhered to the surface bacterial negatively charged molecules that harm the bacterial cell membrane leading to misfunction in its permeability, and finally give rise to cell death **(Kadian et al., 2020)**. Antibiotic-resistant bacteria requires an improvement of new therapeutic methodologies to conquer the shortcoming of traditional antibiotics **(Cano et al., 2020)**. Therefore, the present work focuses on the development and combination of chitosan nanoparticles (CSNPs) and CaPNPs with potent antimicrobial compounds that can aid in delivery of antibiotics to the target sites of drug-resistant microorganisms.

MATERIAL AND METHODS

Chemicals

Chemicals were obtained from Oxoid (Oxoid Ltd., England). Microbial culture media was obtained from Difco Laboratories (Detroit, MI). Fluconazole (Diflucan) and Penicillin G (Pfizerpen) were obtained from Pfizer Corp, New York NY..

Microorganisms

The *Streptomyces rimosus* strain was isolated from the soil (Damietta, Egypt) and identified classically according to the Bergey's Manual of Systematic Bacteriology **(Goodfellow et al., 2012)**. The bacterial and fungal strains used for the antimicrobial activity tests (*Staphylococcus aureus* ATCC25923, *Bacillus cereus* ATCC6633, *Escherichia coli* ATCC25922 and *Candida albicans* ATCC10231) were obtained from Damietta University (The collection culture of Microbiology lab, Faculty of Science).

Experimental procedure

Optimization of the growth factors of the tested isolates

Different environmental factors were tested such as specific incubation times intervals (3, 5, 7and 9 days), different pH (4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0), different temperature (20, 25, 30 and 37°C) and different carbon sources (starch, glucose, glycerol, sucrose, lactose, chitin and cellulose; 20 gm/l) and equimolecular weights from nitrogen sources (potassium nitrate; KNO_3 (2.0 gm/l), ammonium sulphate; $(NH_4)_2SO_4$ (1.96 gm/l), sodium nitrate; $NaNO_3$ (2.5gm/l), peptone (2.9gm/l), casein (2.58gm/l), beef and yeast extract (2.9gm/l). The antimicrobial potential of *S. rimosus* crude metabolite was tested against Gram-negative, Gram-positive bacteria and yeast. The best environmental conditions were determined according to the best inhibition zones (mm) **(Gräfe et al., 1982)**.

Extraction of antimicrobial compounds

The antimicrobial compounds were extracted from the crude metabolites of *S. rimosus* using equal volume ratio (1:1) of different solvents successively (diethyl ether, petroleum ether, chloroform, and butanol). Each organic phase was separated individually and then evaporated with rotary evaporator. The resulting residue of each extraction (3 mg) was dissolved in 50 µL of dimethyl sulphoxide (DMSO) for further antimicrobial activity tests.
The best solvent for the extraction of antimicrobial compounds was tested using mixing the filtrate with solvents (1:1) gradually and then separated using separating funnel equal. Small, concentrated discs (5 mm) of the extracted compounds were prepared and put on the surface of nutrient agar medium which inoculated with 0.5 McFarland standard (1-2 × 10^8 CFU/ml) of pathogenic microbes. Inhibition zones were measured in mm **(Nandhini et al., 2018)**.

Gas chromatography mass spectroscopy analysis

The sample chemical composition was achieved according to **Hamed et al. (2019)** method. Trace GC1310-ISQ mass spectrometer (Thermo Scientific, Austin, TX, USA) and TG-5MS capillary column (30 m x 0.25 mm x 0.25 µm film thickness) were used. The dissolvable postponement was 3 min and 1 µl of test was infused consequently utilizing autosampler AS1300 combined with gas chromatography (GC) in the split mode. Electron ionization (EI) mass spectra were gathered at 70 eV ionization voltages over the scope of m/z 40-1000 in full output mode. The parts were distinguished by correlation of their maintenance times and mass spectra with those of WILEY 09 and NIST 11 mass spectral database **(Shackleton and Marcos, 2006; Shackleton, 2008; Joel and Bhimba, 2012)**

Test for the minimal inhibitory concentration

The microbiological test was done to determine the minimum inhibitory concentration (MIC) of the tested antimicrobial extracts against selected pathogenic strains **(Clinical Laboratory Standards, 2008 and 2017)**. Several test tubes of sterile Luria-Bertani (LB) and Bacto casitone media were prepared for bacteria and yeast, respectively. Different dilutions of the tested extract, Penicillin G and Fluconazole (0.025 to 1 ml) with concentration ranged from (0.15 to 6 mg/ml) was added and then inoculated with 100 µl of 0.5 McFarland standard from the tested microbes. All test tubes were incubated at 37°C for 24 hr and the optical density (OD) of microbial growth measured at 600 nm. Similarly, controls were prepared sole of the extract. MIC was expressed as mg/ml of the antimicrobial agent.

The minimum bactericidal concentration and minimum fungicidal concentration

The minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were detected by subculturing the tested dilutions that inhibited the growth of the tested microbes in the MIC assay. The concentration of 3 mg/ml and 6 mg/ml for *E. coli*, 1.5 gm/ml and 3 mg/ml for *S. aureus*, *B. cereus* and *C. albicans* were inoculated into a fresh extract-free solid medium of LB and Bacto casitone media for bacteria and yeast, respectively. The agar plates were incubated at 37°C for 24 hr. The dilution that produced no single bacterial colony on a solid medium was considered as MBC and the dilution that produced no single fungal colony on a solid medium was considered as MFC **(Akinyemi et al., 2005)**.

Preparation and characterization of nanoparticles

According to **de Moura et al. (2008)** and **Hasaneen et al. (2014)**, CSNPs were prepared by polymerization of meth-acrylic acid (MAA) in CS solution. About 0.2 g of CS were dissolved in 0.5% (v/v) MAA aqueous solution, on a magnetic stirrer for about 12 hr. Then, 0.2 mmol of potassium persulfate were added with continuous stirring at 70°C for 1 h. until CSNPs formation. Finally, the solution was cooled in an ice bath to stop the reaction, centrifuged for 30 min. at 4000 rpm and discarded the supernatant while, the particles were re-suspended in water. The formation of CSNPs occurs through intramolecular linkages between amino groups of CS and polymethacrylic acid (PMAA) carboxyl groups. As a result of CSNPs preparation, the solution of CS in MAA changed from a clear to an opalescent suspension.
CaPNPs were prepared by adding 7.5 ml of $CaCl_2$ (12.5 mM) dropwise to 1 ml of bovine serum albumin (BSA) solution (1 mg/ml) with continuous stirring followed by dropwise addition of 1.5 ml of sodium citrate (10 mM) and 7.5 ml of Na_2HPO_4 (12.5 mM). The solution was stirred for 3 hr. The prepared suspension was centrifuged at 10000 rpm for 20 min. The pellet was washed three times with 0.1 M phosphate buffer (pH 7.2) and re-suspended in 1 ml of phosphate buffer **(He et al., 2000; Joyappa et al., 2009)**.
For further analysis, the nanosolutions were centrifuged (10000 rpm for 15 min) and washed with distilled water to remove excess solution, and then NPs powder was centrifuged and dried at 60°C for 24 hrs.
Shape and size of CSNPs and CaPNPs were identified by using transmission electron microscopy (TEM) analyses (JEOL, JEM-2100, Japan). Zeta potential, a measure of the stability of the prepared NPs, was measured by using by Zeta Potential Analyser (Malvern Zetasizer Nano-ZS90, Malvern, UK). Zeta potential of the prepared NPs either alone or after loading with the selected antimicrobial fraction was measured at 25°C in 12 Zeta runs and a clear dispersal Zeta cell. Data obtained were analysed using Malvern Zetasizer software.

Preparation of nano-loading agents

According to **Yu et al. (2014)** and **Hasaneen et al. (2016)**, loading of the antimicrobial compounds on the surface of either CSNPs or CaPNPs was performed by adding 10 ml of the antimicrobial extract to 20 mg of each NPs and stirred for 6 hr at room temperature.

The antimicrobial activity assay for the loaded nanoparticles with extracted compounds

About 100 µl of the selected antimicrobial fraction, NPs, the selected antimicrobial fraction loaded on the NP and Penicillin G or Fluconazole at concentration 50 µg/ml were added in 0.5 mm pore of the inoculated agar plates with the tested microorganisms. Inhibition zones were measured in mm after 24 h of the incubation at 37°C.
The fold area was determined the solo antimicrobial compound inhibition zones and combined with NPs according to the equation $(B^2 - A^2)/A^2$, A and B; inhibition zones for antimicrobial compound alone and combined with NPs, respectively **(Birla et al., 2009)**.

The releasability and stability of the compound/NPs

The dissolvability of compound/NPs was tried in various solvents like water, ethanol, methanol, n-butyl liquor, dimethylformamide (DMF), $CH_3)_2CO$, hexane and toluene. The Zeta normal size (Zavg) and poly dispersity file (PDI) were determined using the Malvern Zetasizer Nano-ZS90, Malvern, UK.
The dialysis method was used to examine compound/NPs releasability. 5 ml of compound/NPs was put in dialysis tubes (Spectra/Por Biotech; cellulose ester; MWCO 100,000) and afterward sank in 200 mL of distilled water at 37°C. The NPs concentration was estimated by the atomic spectrometer (PerkinElmer, PinAAcle-500, UK).

Statistical analysis

Experimental tests were recorded in triplicate. Results were performed in the form of mean values ± standard deviation (SD). In addition, the results were analysed using SPSS version 18 with statistically significance p-value = 0.05. Consequently, the standard error (SE) was detected.

RESULTS

Optimization of the antimicrobial compounds production by *S. rimosus* isolate

S. rimosus was grown on starch-nitrate liquid medium for 3, 5, 7 and 9 days. Cultures were filtered through filter paper-Whatman's No. 1 and the filtrates were tested against the pathogenic microbes. The results showed that the best incubation period for the production of antifungal and antibacterial was the third day (Table 1).

Table 1 Antimicrobial potential of *S. rimosus* filtrate at different incubation periods against the pathogenic microbes.

Incubation period (Days)	Zone of inhibition (n=3, mm, mean ± SE)			
	B. cereus ATCC6633	*S. aureus* ATCC25923	*E. coli* ATCC25922	*C. albicans* ATCC1023
3	24 ± 0.06	14 ± 0.06	12 ± 0.03	18.6 ± 0.13
5	16 ± 0.06	13.6 ± 0.03	10.6 ± 0.03	10 ± 0.06
7	11 ± 0.10	10 ± 0.06	9.3 ± 0.03	9.6 ± 0.07
9	9 ± 0	9 ± 0	9 ± 0	9.3 ± 0.19

The effect of pH was studied by growing *S. rimosus* on starch-nitrate liquid media at different pH values and for 3 days. The results indicated that the best pH for the antifungal and antibacterial production was at pH 7.0 (Table 2).

Table 2 Antimicrobial potential of *S. rimosus* filtrate at different pH values against the pathogenic microbes.

pH value	Zone of inhibition (n=3, mm, mean ± SE)			
	B. cereus ATCC6633	*S. aureus* ATCC25923	*E. coli* ATCC25922	*C. albicans* ATCC1023
4	0 ± 0	0 ± 0	0 ± 0	0 ± 0
5	11 ± 0.03	15.3 ± 0	10.3 ± 0.03	11 ± 0
6	13.6 ± 0.03	16 ± 0	11.6 ± 0.03	10 ± 0
7	26.3 ± 0.03	17.6 ± 0.03	15 ± 0	18.3 ± 0.08
8	21.6 ± 0.06	14 ± 0	12 ± 0	13.6 ± 0.08
9	18 ± 0	14.6 ± 0.03	12 ± 0	13.3 ± 0.03
10	12.6 ± 0.03	11.6 ± 0.03	10 ± 0	12.3 ± 0.03

Different temperatures were tested to determine the best temperature for production of antifungal and antibacterial metabolites by growing *S. rimosus* on starch-nitrate liquid medium, pH 7.0 and incubated for 3 days. The optimum temperature was recorded at 30°C. By increasing the temperature, the inhibition zone decreased as shown in Table 3.

Table 3 Antimicrobial potential of *S. rimosus* filtrate at different temperatures against the pathogenic microbes.

Temperature (°C)	Zone of inhibition (n=3, mm, mean ± SE)			
	B. cereus ATCC6633	*S. aureus* ATCC25923	*E. coli* ATCC25922	*C. albicans* ATCC1023
20	0 ± 0	0 ± 0	0 ± 0	0 ± 0
25	20.3 ± 0.03	16.3 ± 0.03	15 ± 0.05	13.6 ± 0.06
30	23.6 ± 0.01	18 ± 0	15.3 ± 0.05	16.6 ± 0.01
35	18.6 ± 0.06	14.6 ± 0.06	12.6 ± 0.03	15.6 ± 0.03
40	12.3 ± 0.03	10.3 ± 0.03	11 ± 0	12.6 ± 0.01

Different carbon sources test showed that the starch was the best carbon source for antimicrobial metabolites production followed by sucrose, glucose and lactose (Table 4).

Table 4 Antimicrobial potential of *S. rimosus* filtrate at different carbon sources against the pathogenic microbes.

Carbon source	Zone of inhibition (n=3, mm, mean ± SE)			
	B. cereus ATCC6633	*S. aureus* ATCC25923	*E. coli* ATCC25922	*C. albicans* ATCC1023
Starch	21 ± 0.06	25 ± 0.06	17.3 ± 0.03	18.6 ± 0.06
Glucose	13.6 ± 0.06	12 ± 0	10.6 ± 0.03	15 ± 0
Sucrose	16.7 ± 0.03	21 ± 0.06	15.3 ± 0.03	15.3 ± 0.06
Lactose	10.6 ± 0.03	11.3 ± 0.03	0 ± 0	0 ± 0
Cellulose	0 ± 0	0 ± 0	0 ± 0	10 ± 0
Chitin	10 ± 0	5.6 ± 0.03	0 ± 0	0 ± 0
Glycerol	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Effect of different nitrogen sources experiment showed that KNO_3 was the greatest nitrogen source for the production of antifungal and antibacterial compounds followed by peptone, $NaNO_3$, beef extract and casein for Gram-positive bacteria while it was peptone followed by casein, beef extract and yeast extract for the Gram-negative *E. coli*. The optimum activity of *S. rimosus* filtrate was at using casein as nitrogen source followed by KNO_3, peptone, beef extract, $NaNO_3$ and yeast extract (Table 5).

Table 5 Antimicrobial potential of *S. rimosus* filtrate at different nitrogen sources against the pathogenic microbes.

Nitrogen source	Zone of inhibition (n=3, mm, mean ± SE)			
	B. cereus ATCC6633	*S. aureus* ATCC25923	*E. coli* ATCC25922	*C. albicans* ATCC1023
KNO_3	22 ± 0.06	16.6 ± 0.03	0 ± 0	13.6 ± 0.03
$NaNO_3$	16.3 ± 0.03	12.3 ± 0.03	0 ± 0	10.3 ± 0.03
$(NH_4)_2SO_4$	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Peptone	17.6 ± 0.03	14.3 ± 0.06	16 ± 0.06	13.6 ± 0.06
Beef extract	15.3 ± 0.03	12.3 ± 0.03	12.3 ± 0.03	12.6 ± 0.03
Yeast extract	0 ± 0.03	13.3 ± 0.03	10.6 ± 0.06	10.3 ± 0.03
Casein	14.6 ± 0.06	14.3 ± 0.06	12.4 ± 0.03	14.6 ± 0.06

After the separation and extraction of crude metabolites using different organic solvents such as diethyl ether, petroleum ether, chloroform and butanol on the rotary evaporator, small, concentrated discs from each extract were prepared and tested against the pathogenic microbes. The best solvent for the extraction of antimicrobial compounds was diethyl ether as showed in Table 6.

Table 6 The best solvent for extraction of antimicrobial compounds.

Solvent	Zone of inhibition (n=3, mm, mean ± SE)			
	B. cereus ATCC6633	*S. aureus* ATCC25923	*E. coli* ATCC25922	*C. albicans* ATCC1023
Diethyl ether	18.3 ± 0.06	17 ± 0	11.3 ± 0.06	18 ± 0
Petroleum ether	12 ± 0	12.6 ± 0.06	10 ± 0	13 ± 0
Chloroform	10.6 ± 0.03	11.6 ± 0.03	9.3 ± 0.03	12 ± 0
Butanol	11 ± 0.06	11.6 ± 0.03	11.6 ± 0.03	16 ± 0.03

Gas chromatography-mass analysis for the diethyl ether extract

The components present in the extract of *S. rimosus* were identified by gas chromatography-mass (GC-MS). The chromatogram was showed in Figure 1. These components were arranged according to their active principles with their retention time (RT), percentage composition in the sample, molecular formula, and molecular weight (MW) was presented in Table 7. In this sample, nine antimicrobial components were identified and considered as organic acids as follow: 1,2-Benzenedicarboxylic acid, Dioctyl Ester (10.93%) as the major component followed by 9- Octadecenoic acid (10.75%), E,E,Z-1,3,12-Nonadecatriene-5,14-d Iol (9.42%), 9-Hexadecenoic acid,9-octadecenyl ester, (Z,Z)- (9.26%), Heptatriacotanol (8.56%), Tributyl acetylcitrate (8.18%), Z-(13,14-Epoxy)tetradec-11-en-1-ol acetate (7.79%), 1,3,5-Triaziene-2,4-diamine,6-Chloro-N-Ethyl (0.55%) and 1,4-Methanoazulene and Decahydro-4,8,8-Trimethyl-9-Methylene- (0.48%) (Figure 1).

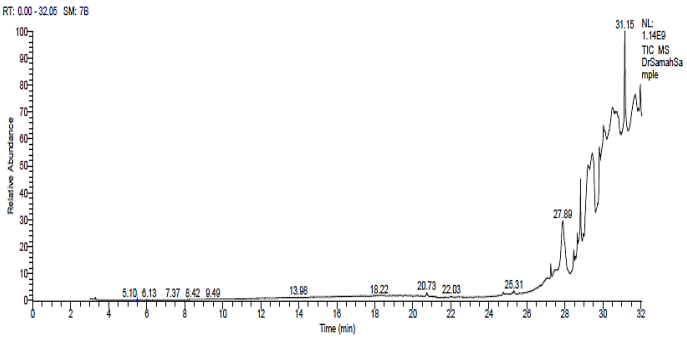
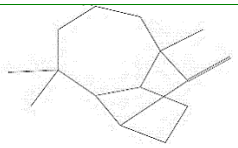
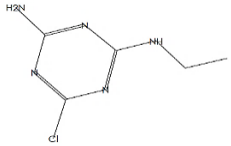
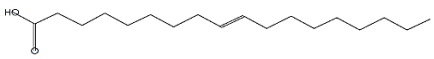
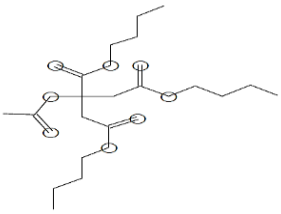
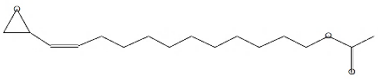
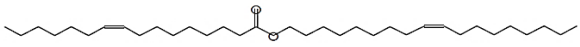
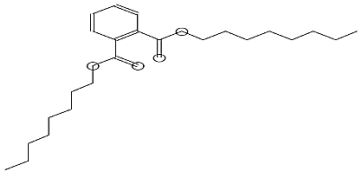
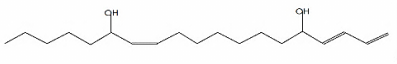


Figure 1 GC-MS chromatogram of the extracted diethyl ether filtrate of *S. rimosus*.

Table 7 Components detected in the diethyl ether extraction of S. *rimosus* by GC-MS analysis

Peak	RT	Compound name and structure	Area %	Molecular Formula	Molecular Weight
1	20.73	 1,4-Methanoazulene, decahydro-4,8,8-trimethyl-9-methylene	0.48	$C_{15}H_{24}$	204
2	25.31	 1,3,5-Triaziene-2,4-diamine,6-Chloro-N-Ethyl	0.55	$C_5H_8ClN_5$	173
3	27.89	 9- Octadecenoic acid (z)	10.75	$C_{18}H_{34}O_2$	282
4	28.82	 Tributyl acetylcitrate	8.18	$C_{20}H_{34}O_8$	402
5	29.20	1-Heptatriacotanol	8.56	$C_{37}H_{76}O$	536
6	29.46	 Z-(13,14-Epoxy)tetradec-11-en-1-ol acetate	7.79	$C_{16}H_{28}O_3$	268
7	30.03	 9-Hexadecenoic acid,9-octadecenyl ester, (Z,Z)	9.26	$C_{34}H_{64}O_2$	504
8	31.15	 1,2-Benzenedicarboxylic acid, Dioctyl ester	10.93	$C_{24}H_{38}O_4$	390
9	31.68	 E,E,Z-1,3,12-Nonadecatriene-5,14-d Iol	9.42	$C_{19}H_{34}O_2$	294

Charactarization of NPs and the loaded NPs on the antimicrobial compounds

CSNPs were appeared as spherical shaped NPs with a homogeneous size in distribution and diameter approximately = 22 ± 2 nm. The mean diameter of CSNPs was increased after loading with the antimicrobial compounds but still had a spherical shape with diameter ranged from 55 up to 100 nm. (Figure 2A). CSNPs loaded with antimicrobial molecules diameter was approximately 55 upto 100 nm (Figure 2B).

CaPNPs had also a homogeneous spherical shape with a size diameter range from 70 to 95 nm (Figure 2C). Loading of antimicrobial compounds onto CaPNPs made them appeared in rod shape with width varying from 14 to 27 nm and length from 274 to 397 nm (Figure 2D).

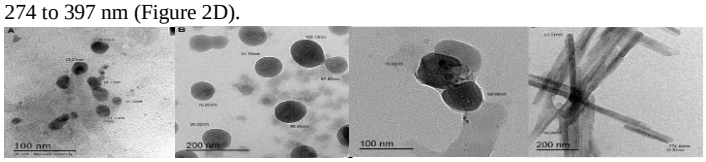


Figure 2 TEM micrograph of CSNPs; (A), CSNPs loaded with the antimicrobial compounds; (B), CaPNPs; (C) and CaPNPs loaded with the antimicrobial compounds; (D).

The values of Zeta potential stand for stability of NPs either alone or after loading them with the antimicrobial compounds (Table 8), where the more negative values can be considered as disability index of NPs preparation. Zeta Potential and electrical conductivity (EC) were increased by loading of the antimicrobial compounds on the NPs surfaces. The variation in the Zeta potential of the two NPs might be attributed to the difference in energy of electrical field in both CSNPs and CaPNPs surface surrounded by another ion.

Table 8 Zeta potential and EC of CSNPs and CaPNPs either alone or loaded with the antimicrobial compounds.

Nanomaterial	Zeta potential	EC (mS/cm)
CSNPs	-7.48 ± 0.19	3.22 ± 0.13
Loaded CSNPs	-7.93 ± 0.03	3.52 ± 0.06
CaPNPs	-14.03 ± 0.48	1.62 ± 0.07
Loaded CaPNPs	-22.87 ± 1.92	8.48 ± 0.05

Antimicrobial activity of *S. rimosus* diethyl ether extract

The MIC of the diethyl ether extract of *S. rimosus* was 3 mg/ml for *E. coli* and 1.5 mg/ml for *S. aureus*, *B. cereus* and *C. albicans*. The MBC values of diethyl ether

extract of *S. rimosus* were 6.0 mg/ml for *E. coli* and 3.0 mg/ml for *S. aureus* and *B. cereus*. The MFC of *S. rimosus* extract against *C. albicans* was recorded at 3.0 mg/ml. While Penicillin G showed MIC value 6.25 mg/ml against *S. aureus*, *B. cereus* and *E. coli*. Fluconazole inhibited *C. albicans* at 1.5 mg/ml.

Antimicrobial activity of *S. rimosus* diethyl ether extract loaded on NPs

The tested pathogenic microbes were resistant to the prepared NPs. On the other hand, the compound itself showed good activity which can be directly used for antimicrobial application but the loading process of the antimicrobial compounds on the synthesized NPs increased its activity (Table 9). This increasing occurred by the combination of the antimicrobial compound with NPs that increased fold areas especially against *B. cereus*, *S. aureus* and *E. coli* (Table 10). The less inhibition effects of antimicrobial compound (13, 13.3 and 13.6 mm) were against *S. aureus*, *C. albicans* and *B. cereus*, respectively. Conversely, the antimicrobial compound showed a good antimicrobial action when companied with NPs showing higher fold areas.

Table 9 Antimicrobial activity of *S. rimosus* extract after loading on the NPs.

Antimicrobial agent	Zone of inhibition (n=3, mm, mean ± SE)			
	B. cereus ATCC6633	*S. aureus* ATCC25923	*E. coli* ATCC25922	*C. albicans* ATCC1023
Extract	14.0 ± 0.03	13.0 ± 0.03	13.6 ± 0	13.3 ± 0
CSNPs	ve-	ve-	ve-	ve-
CSNPs + extract	17.5 ± 0	17.3 ± 0.06	22.2 ± 0.03	20.0 ± 0
CaPNPs	ve-	ve-	ve-	ve-
CaPNPs + extract	16.5 ± 0.03	18.5 ± 0.03	25 ± 0.03	14.2 ± 0
Penicillin G	30.2 ± 0	10.0 ± 0.14	12.0 ± 0	-
Fluconazole	-	-	-	11.0 ± 0.03

Table 10 Antimicrobial action of the companied antimicrobial compound with NPs.

Bacteria	Zone of inhibition (n=3, mm, mean)			Increase in fold area♦	
	The antimicrobial compound (*A*)	CSNPs & compound (*B*)	CaPNPs & compound (*B'*)	CSNPs & compound (*B*)	CaPNPs & compound (*B'*)
E. coli	14	17.5	16.5	0.56	0.39
S. aureus	13	17.3	18.5	0.77	1.03
B. cereus	13.6	22.2	25	1.66	2.38
C. albicans	13.3	20	14.2	1.26	0.14

♦ $(B^2 \text{ or } B'^2 - A^2) / A^2$, where A and B or B' are the zones of inhibition for the antimicrobial compound and NPs & compound, respectively.

The releasability and stability of the compound/NPs

The compound/NPs was stable in the several solvents for 3 h. The compound/NPs showed a well diffusion in high polar solvents (ethanol and DMF) but low diffusion in nonpolar solvents (hexane and toluene). The dialysis experiments showed that the very slow diffusion rate of NPs (Figure 3). PDI and Zavg for compound/NPs were shown in Table 11.

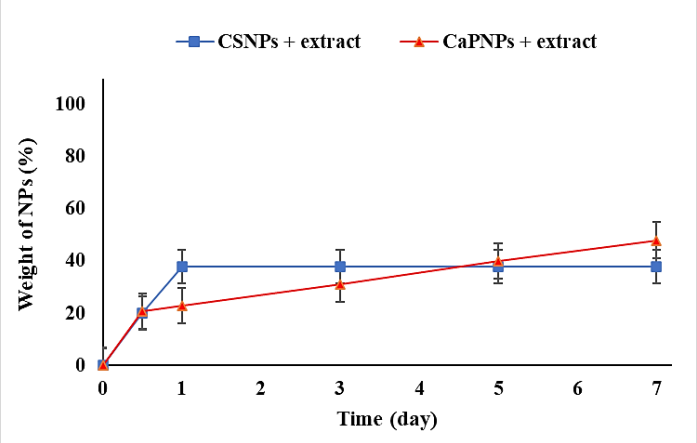


Figure 3 The release property of compound/NPs at 37°C.

Table 11 The PDI and Zavg for compound/NPs in different solvents.

Solvent	PDI (d.nm)		Zavg (nm)	
	CSNPs & compound	CaPNPs & compound	CSNPs & compound	CaPNPs & compound
Water	0.421	0.332	324	233
Methanol	0.8	0.7	850	742
Ethanol	0.7	0.6	471	387
DMF	0.5	0.4	397	277
n-butyl alcohol	0.5	0.4	336	306
Acetone	0.8	0.7	1643	1522
Toluene	0.9	0.8	1177	1007
Hexane	0.6	0.5	632	595

DISCUSSION

Actinomycetes are one of the most widely spread group of microbes in **environment (Oskay et al., 2004)**. About 75% of the familiar traditional and therapeutically antibiotics are yielded by *Streptomyces* **(Sujatha et al., 2005)**. On the other hand, several reports found that these microbes are obvious by the naked eye as colonies of about 1-10 mm in diameter **(Groth et al., 1999)**. The majority of actinomycetes widely distributed in soil and the genus *Streptomyces* is the biggest producer of antibiotics. *Streptomyces* sp. had been well documented for industry applications for production of bioactive molecules for example vitamins, antibiotics, and enzymes **(Laorpaksa et al., 1987)**.

Antimicrobial resistance is considered as a worrying problem for the public health and agriculture sectors especially strains such as *E. coli*, *S. aureus*, *B. cereus* and *C. albicans* **(El-Zahed et al., 2021)**. So, new approaches should renovate to handling the resistance development. Recently, antimicrobial activity of nanomaterials such as CSNPs and CaPNPs had been developed as a response for this concern **(Rao et al., 2020)**.

The capability of *Streptomyces* to produce the bioactive molecules is not a fixed ability nevertheless could be increased or lost by controlling nutrition conditions and cultivation **(Saadoun and Gharaibeh, 2003)**. Improvement the growth and antibiotic assembly can be obtained by operating the nutritious and physical factors of the culturing settings, so the component of media shows a fundamental role in the productivity and financial side of the final progression. Changes in the some cultivation factors like incubation period, pH, and temperature had a chief role in the production process **(Kiranmayi et al., 2011)**. Furthermore, the change in carbon and nitrogen sources had reported to assume antibiotic production using *Streptomyces* **(Barratt and Oliver, 1994; Reddy et al., 2011).**

In this study, *S. rimosus* antimicrobial filtrate was selected, optimized, loaded on CSNPs and CaPNPs and then examined against bacteria and yeast. Different parameters were tested for obtaining the best conditions for the production of potent antimicrobial compounds. The present study showed that the best incubation time to produce antifungal and antibacterial compounds was the third day. *Streptomyces* BT-408 gave a maximum activity of its antimicrobial metabolite at the fourth day as recorded by **Sujatha et al. (2005)**. Furthermore, **Narayana and Vijayalakshmi (2008)** observed that the production of antibiotic using *Streptomyces albidoflavus* started after the third day and reached maximum at the fifth day which represented as stationary phase of incubation period. It is known that the culture medium pH is the most significant factors, as it is an obvious effect on the enzyme's activity and their metabolic actions, in addition to the major influence on complex physiological occurrences such as cell morphology and membrane permeability **(Guimarães et al., 2004)**. The results revealed that the best pH value for the production of strong antimicrobial compounds was at pH 7.0 to pH 8.0. The neutral pH value was the most suitable for maximum assembly of metabolites **(Ripa et al., 2009)**. On the other hand, the extreme pH is unsuitable for production of secondary metabolites. Neutral pH has main role in the growth of *Streptomyces* sp and production of secondary metabolite **(Bhavana et al., 2014)**. Normally, *Streptomyces* are sensitive to temperature **(Devi et al., 2015)**. The temperature is one of the most important factors which affect the growth of *Streptomyces* and production of bioactive compounds. After studying the effect of different temperatures on growth and bioactive metabolite production of *S. rimosus*, the results indicated that the best temperature for antimicrobial action was 30°C. Extreme temperatures are generally inappropriate for the bioactive metabolite production as the harmful effect moved on the enzymes involved in the biosynthetic pathway **(Ripa et al., 2009)**. Sugars and nitrogen play important roles as energy and structural compounds in cell. The cell metabolism of *Streptomycetes* at an excess nutritional led to the cell mass generation instead of secondary metabolites production. The tested *S. rimosus* showed maximum cell growth in medium amended with starch which considered a complex carbon source for *Streptomyces* as recorded by **Huck et al. (1991)**. Starch and maltose used quickly to produce cellular components, so that; these carbon sources would be used as energy source for antibiotic production **(AL-Mawlah, 2018)**. Glucose and lactose gave lowly production of bioactive metabolite, conversely it was found that cellulose and glycerol gave negative results and caused a catabolic repression. Starch was the best carbon source for the production of antibiotic **(El-Naggar et**

al., 2003; Osman et al., 2011). Accordingly, the variance of carbon sources of media leads to variable levels of antimicrobial metabolite. Nitrogen source accommodating is the key factor in the regulation of antibiotic production in microorganisms **(Voelker and Altaba, 2001)**. For investigating the effect of nitrogen source on the antimicrobial metabolite production from *S. rimosus*, different nitrogen sources were investigated. In comparison of some inorganic nitrogen sources and organic nitrogen sources it was found that potassium nitrate which considered inorganic nitrogen source was the optimum nitrogen source on bioactive metabolic production. Peptone has been found to favour antibiotic production as reported by **Chattopadhyay and Sen (1997) and Praveen et al. (2008)**. Finally, results confirmed that the growth of *S. rimosus* was significantly affected by the type and nature of nitrogen source in the medium. The availability and nature of nitrogen from the inorganic and organic nitrogen compounds in fermentation media detected different effect on the antibiotic levels. High yields of antibacterial compounds are influenced by a low temporal coupling between antibacterial compounds synthesis and biomass accumulation.

The International Union of Pure and Applied Chemistry (IUPAC) recommend using the liquid-liquid dispersal over the conventional term solvent extraction **(Rydberg, 1992)**. In this study, the crude extract was obtained by using four different solvents (diethyl ether, petroleum ether, chloroform, and butanol) respectively. The antimicrobial activity of diethyl ether fraction of the crude extract was most effect against *B. cereus*. The other solvents (petroleum ether, chloroform, and butanol) behaved lower antimicrobial activity. Diethyl ether is considered a good organic extracting solvent because it has a low polarity. Also, diethyl ether is used as an organic separator compound due to its natural material sources. Besides, diethyl ether is standard to anaesthesia in medical applications. In addition, diethyl ether is used as an organic solvent in many reactions **(Butt et al., 1962)**.

Recently, GC-MS studies have been increased gradually for the analysis medical used components **(Nandagopalan et al., 2015)**. In this study the GC-MS analysis showed multiple compounds from the diethyl ether extract from *S. rimosus*. These compounds are responsible for the anticandidal and antibacterial activity. Nine components were identified in the isolate of *Streptomyces* species. These compounds were 1,2-Benzenedicarboxylic acid, Dioctyl Ester (10.93%) as the major component followed by 9- Octadecenoic acid (10.75%), E,E,Z-1,3,12-Nonadecatriene-5,14-d Iol (9.42%), 9-Hexadecenoic acid,9-octadecenyl ester, (Z,Z)- (9.26%), Heptatriacotanol (8.56%), Tributyl acetylcitrate (8.18%), Z-(13,14-Epoxy)tetradec-11-en-1-ol acetate (7.79%), 1,3,5-Triaziene-2,4-diamine,6-Chloro-N-Ethyl (0.55%) and 1,4-Methanoazulene,Decahydro-4,8,8-Trimethyl-9-Methylene- (0.48%). The biological activities of the components were identified according to the antimicrobial, antioxidant and antitumoral activities, beside that the nature of these compounds are mostly organic acids **(Gálvez-Iriqui et al., 2019; Gopinath et al., 2020; Ulu et ak., 2021)**.

Adjuvants compounds play significant role in improving the effectiveness and immunogenicity of a drug in particular antibiotics and vaccines. Recently, the NPs research has paved away towards the development of improved and advanced antibiotics and vaccines. Adjuvants inorganic as well as organic compounds have recently been explored and exploited in NPs scale. They have been found to have great significance in entrapping and binding biomolecules of medical and veterinary importance and have proved to be more advantageous as compared to macromolecular forms in being inexpensive and easy to produce. Further, the bioavailability of the biomolecules has also been found to significantly increase when formulated in nano scale. Hence, those nanoparticles play major role in numerous fields like gene therapy, adjuvants and drug delivery systems **(Joyappa et al., 2009).** In this study, we have screened the probability to use CSNPs as delivery systems with antibiotic activity. CS is the second most abundant polymer in nature that found in invertebrates' structures and in the fungal cell walls **(Duttagupta et al., 2015)**. CS is comprising of copolymers of β-1,4-linked N-acetyl-D-glucosamine and D-glucosamine achieved by deacetylation of chitin. CSNPs showed a variable pattern release in the different stage. It includes that drug absorbing on the surface of NPs separated into the releasing medium rapidly, resulting in the burst release of drug in the initial stage. By degradation and diffusion, the drug moved through inside pores in NPs slowly and fell into the medium by diffusion. The formation of CSNPs occurs through intramolecular linkages between amino groups of CS and PMAA carboxyl groups which known as polymerization of MAA. After CSNPs preparation, the solution of CS in MAA changed from a clear to an opalescent suspension, this is due to formation of CSNPs with MAA. This compatible with the mechanism which made by **De Vasconcelos et al. (2006)**. Lately, the antimicrobial activity of 1,2-Benzenedicarboxylic acid, dioctyl ester (BCA) had been described as *S. griseus* secondary metabolite and used in the biological control experiments **(Gálvez-Iriqui et al., 2019)**. In the present study, BCA was found as the major component of *S. rimosus* extract. The antimicrobial action of CS depends on chemical properties for example electrostatic interactions between the amino groups of CS and the stimulation of the destabilization of the plasma membrane **(Chung and Chen, 2008; Xing et al., 2015)**. These mechanisms had been reported against bacteria, yeasts, and moulds **(Teodoro et al., 2014; Chávez-Magdaleno et al. 2018)**. **Yien et al. (2012)** stated that *A. niger* was resistant to CSNPs. Similarly, our results revealed that CSNPs did not show any antimicrobial property but when combining with the extract efficiently showed stronger antimicrobial activity compared to the extract alone. The BCA antimicrobial activity mechanism has not been clarified.

CaPNPs are safe particles at the size of 50-100 nm **(Joyappa et al., 2009)**. These NPs are beneficial for DNA mucosal immunity and vaccines **(Mody et al., 2013; He et al., 2000)**, and show a good biocompatibility. Calcium phosphate nanoparticles (CAPs), potentially present in the most natural choice for antibiotic delivery platforms in bone therapy. Firstly, CaPNPs is the natural mineral component of bone in the form of crystals in bone, stiff but brittle, this is useful for imparting sufficient compressive strength to intrinsically tough collagen fibers. In addition, CaPNPs are a bioactive and osteo-conductive to bone growth. Not only that, but also it considered most bio-compatible nanomaterials assessed for toxicity **(Singh and Lillard, 2009)**. CaPNPs were prepared by precipitation from aqueous solutions: flaky, brick-like, spherical and elongated orthogonal. CaPNPs are the most commonly used adjuvant and delivery system that was first developed by **He et al. (2002)**. The interaction between cells and particles utilized for drug delivery is critically depends on their chemical composition and other parameters like size, shape, surface, charge, crystallinity and porosity **(Gratton, et al., 2008)**. CaPNPs are considered as hard material NPs, which have usually used in drug delivery **(Xiang et al., 2021)** and have thus been designed to stimulate antigen connection **(Wendorf et al., 2006)**. Zeta Potential values of CSNPs and CaPNPs are considered as an important indicator for the stability of the two engineered nanomaterials. The variation in the ratio of Zeta potential can be attributed to the energy electrical field in both CSNPs and CaPNPs surface surrounded by another ion. The negative potential of the NPs loaded with the antimicrobial compounds approved the long-term stability and high dispersity.

The diethyl ether fraction had the broadest antimicrobial action in MIC, MBC and MFC against the pathogenic microbes. The extracted antibiotic showed antimicrobial spectrum against all the tested pathogenic microorganisms. Principally it is shown that the antimicrobial effect of NPs depends on its size and morphology, and this means that as size decreasing from 10 µm to 10 nm as the surface area increased multifold, so the antimicrobial effect also increased multifold **(Birla et al., 2009)**. In this work, compound/NPs was stable, with a slow releasement rate and a good dispersion of the solute in the solvent due to its small PDI and Zavg (0-1). The solubility of compound/NPs were tested in different solvents **(Danaei et al., 2018)**. In addition, the compound/NPs showed a good dispersion in DMF, water and n-butyl alcohol (PDI<0.5 d.nm, Zavg <400 nm). Also, the negative charge of the compound/NPs increased its dispersity and led to long-term stability preventing its aggregation.

The activity of this antibiotic was studied and matched with the results that reported by **Rambali et al. (2001)**. Accordingly, we could conclude that the extracted compounds had abroad antimicrobial spectrum, and further study will be designed to elucidate the mode of action of the NPs loaded with the antimicrobial compounds as an antimicrobial agent.

CONCLUSIONS

Streptomyces rimosus crude antimicrobial metabolite was loaded on CSNPs and CaPNPs as a cost-effective and developed approach for antibiotics resistance problems. The crude antimicrobial metabolite included nine potent antimicrobial organic acids that confirmed using gas chromatography-mass. This method provides antimicrobial compounds that loaded on CSNPs and CaPNPs owning competitive size, shape and antimicrobial activities Thusly, *S. rimosus* could be developed as a nano-biofactory and drug delivery systems against human pathogenic microbes.

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