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Nanoencapsulation of mānuka oil and β-triketones for antimicrobial delivery in oral wounds

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ABSTRACT

Objectives: Bacterial invasion of periodontal tissues is a common oral health concern, highlighting the need for novel antimicrobial agents. In this study, we aimed to nanoencapsulate mānuka oil and its purified *β*-triketones in chitosan and evaluate the antimicrobial potential of these formulations for oral applications.
Methods: Chitosan nanoparticles were produced containing mānuka oil and its purified *β*-triketone components. The ratios of chitosan to sodium tripolyphosphate (TPP) of 5:1, 4:1, and 3:1 were investigated at two pH levels for chitosan and TPP. Nanoparticles were investigated for size and uniformity (polydispersity index: PDI). The antimicrobial activity of the produced nanoparticles was evaluated against *E. faecalis*, *S. mutans*, *P. gingivalis,* and *S. aureus*. The nanoparticles were assessed for yield, encapsulation efficiency (EE), and biocompatibility, and were visualized using light and scanning electron microscopy.
Results: The optimal conditions for producing nanoparticles were a chitosan: TPP ratio of 5:1, with chitosan adjusted to pH 5.5 and TPP maintained at its unadjusted pH 9. The particle sizes were 327 nm (PDI: 0.654), 336 nm (PDI: 0.471), and 288 nm (PDI: 0.379) for unloaded chitosan, chitosan encapsulating mānuka oil, and purified *β*-triketone, respectively. Freeze-dried yield was 266-370 mg/ml with a high EE of 89%. The release profile for mānuka oil showed 91% by day 5, while for *β*-triketone, 93% was released by day 7. Mesenchymal stem cells showed reduced metabolic activity with >6.25 mg/ml of nanoparticles, regardless of payload.
Conclusions: Mānuka oil and *β*-triketone nanoparticles were produced with promising attributes as antimicrobials for intraoral application.

1. Introduction

Natural products have been used for many years as a source of active ingredients for therapeutic applications [1]. The use of natural based antimicrobial compounds has gained greater interest in recent years due to increasing rates of antibiotic resistance [2]. Additionally, natural compounds are thought to be less cytotoxic to human cells compared to synthetic alternatives [3].

Mānuka or *Leptospermum scoparium* of the *Myrtaceae* family is a native shrub of New Zealand and Australia. Māori, the indigenous peoples of New Zealand, refer to mānuka as a taonga or 'treasure', and have an established history of using it as both a traditional medicinal and as a food [4]. Leaf decoctions were traditionally used to reduce fever and treat colds, while bark preparations were used as sedatives [5]. Mānuka oil extracts were used for treating diarrhea, colds and inflammation [6]. The antimicrobial activities of *L. scoparium* essential oil have been attributed to the presence of 3 particular *β*-triketones: flavesone, leptospermone, and iso-leptospermone [7].

Only a very limited number of formulations have been attempted incorporating mānuka oil in order to retain its antimicrobial properties and provide substantivity of release; these include emulsions [8] and nano-emulsions [9]. In addition, a commercial mouthwash (GINGINAT)

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has been developed using microencapsulation to allow an *in situ* slow release of several natural active ingredients including mānuka oil over several hours [10]. These formulations have been shown to have improved antimicrobial and antifungal activities compared to the oils alone. However, none of the mentioned formulations were able to provide a release that will not be immediately washed away from the targeted tissue. Moreover, no attempts have been made to incorporate the β-triketones alone in any medicinal formulations. Purified β-triketones can be extracted with 99% purity, having the advantage of three-time higher antimicrobial activity, and the removal of multiple unknown components simplifying product reproducibility and safety.

Chitosan is a natural amino polysaccharide produced from the deacetylation of chitin obtained from crustaceans and insects [11]. This polymer shows both biocompatibility and biodegradability [12]. It has been widely used in the pharmaceutical industry and is considered a novel carrier for drug delivery [13]. Chitosan has been shown to have its own antimicrobial activity [14], as well as having mucoadhesive properties, giving it advantageous properties to enhance substantivity and bioavailability of drugs within in the oral environment [15].

Different methods have been studied to produce chitosan nanoparticles (CNPs) with encapsulation of proteins including crosslinking, drying, sieving, precipitation, and reversed micelles [16]. Using these methods, the particle size, stability of the active component, and toxicity all vary considerably [17]. One of the most widely used methods has been ionic gelation of chitosan using sodium tripolyphosphate (TPP), this method has the advantages of being relatively simple, avoids the use of organic solvents and is conducted at low temperatures thus allowing the encapsulation of unstable molecules [18].

The aim of this study is to investigate the encapsulation of mānuka oil and β-triketones in CNPs as a way of controlling release for oral wounds.

2. Materials and methods

2.1. Preparation of the nanoparticles

Nanoparticles of chitosan alone, chitosan encapsulating mānuka oil, and chitosan encapsulating the β-triketones were produced. The nanoparticles were prepared following the procedure described previously with modifications [18,19]. Chitosan (low molecular weight, 448869-50G, Sigma-Aldrich®, St Louis, USA) 0.35% w/v was stirred overnight in 1% v/v acetic acid (glacial 100%, 1000632500, EMSURE®, Germany), the solution was subsequently centrifuged at 6000 g for 30 min and the supernatant was collected for subsequent use. CNPs were obtained by inducing gelation with sodium tripolyphosphate (TPP) (238503-25G, Sigma-Aldrich®, St Louis, USA). Ionotropic gelation takes place due to interaction between positively charged amino groups and negatively charged TPP. Three different chitosan:TPP final mass ratios were used; 5:1, 4:1, and 3:1. Chitosan pH was either unadjusted (pH 3.9) or adjusted to (pH 5.5) using 1 M NaOH. TPP was dissolved in Milli-Q H_2O and stirred on medium speed for 15 min until completely dissolved. TPP pH was either unadjusted (pH 9) or adjusted to (pH 5.5) using 1 M acetic acid. Tween® 80 (P6224-500 ML, Sigma-Aldrich®, St Louis, USA) was added to the chitosan solution and stirred for 2 h at 50°C to a final concentration of 1.125% v/v. Mānuka oil or β-triketones was subsequently added to the chitosan/Tween 80 mixture and stirred at 25°C for 1 h to a final concentration of 1% v/v. The crosslinking reagent TPP was then added to the chitosan mixture using an Eppendorf dispenser set to deliver 20 ml drops, with fixed volumes of chitosan:TPP of 2.5:1, so the final concentration of chitosan was 0.25%, and the nanoparticles then mixed for a further 30 min. The resultant mix was centrifuged at 12,000 g for 15 min, the top fraction was collected and kept to measure the encapsulation efficiency, and the bottom fraction was collected for recovery of the CNPs. D-Trehalose dihydrate (90210-250G, Millipore®, Germany) 5% w/v was added to the bottom fraction and mixed by vortexing until completely dissolved, to prevent aggregation of the particles and to protect them during lyophilization. The CNPs were kept at −80°C and then freeze-dried.

Gas Chromatographic analysis was conducted for both the mānuka oil and the β-triketones before using in the nanoparticle formations.

2.2. Encapsulation efficiency

Encapsulation efficiency was obtained by determining the concentration of unincorporated mānuka oil or β-triketones after nanoparticle formation, in the supernatant recovered after centrifugation. This was measured spectrophotometrically at 280 nm [18,20]. The amount of the encapsulated oil was calculated as the difference between the Optical Density (OD) of maximum oil concentration (1%) and the oil concentration recovered in the supernatant.

The supernatant of the control chitosan (unloaded nanoparticles; 1 ml) was used as a blank. The same supernatant was used to calculate the maximum oil concentration after adding mānuka oil or β-triketones (1%) and measuring the OD. Encapsulation efficiency (EE%) was calculated using the following equation:

$$EE\% = \text{OD of maximum oil concentration} - \text{OD of sample} \times 100$$

OD of maximum oil concentration.

2.3. Nanoparticle characterization

2.3.1. Dynamic light scattering (DLS)

The average particle size and uniformity of CNPs were measured as described by Rampino et al. [18] using a Malvern Zetasizer Pro Blue Range (ATA Scientific Pty Ltd, NSW, Australia). Size values were reported as mean of Z-average, standard deviation (SD), and polydispersity index (PDI) of four runs. Zeta potential was measured on the optimized nanoparticles.

2.3.2. Light microscopy

Freeze-dried CNPs (25 mg) were resuspended in 300 ml of phosphate buffered saline (PBS; #SALB010, Gibco, MT, USA) and centrifuged at 10,000 g for 5 min at RT. One ml of 0.3% Oil Red O-stain (#O0625-25G, Sigma-Aldrich®, St Louis, USA) in H_2O with a final concentration of 0.4% dextran was added to the pellet and vortexed, then left to stand at RT for 30 min. The nanoparticles were repeatedly washed with 500 ml of PBS and observed with an EVOS M5000 Imaging System.

2.3.3. Scanning electron microscopy

The freeze-dried CNPs were reconstituted in PBS and air dried at RT on copper grids. Samples were viewed and imaged in a Zeiss Sigma 300VP FESEM (Carl Zeiss Inc, Germany) at an accelerating voltage of 5 KV.

2.4. In vitro release of mānuka oil and β-triketones

Chitosan, chitosan/mānuka oil, and chitosan/β-triketones nanoparticles (10 mg each) were incubated in 1100 ml of PBS (pH 7.4) at a constant temperature (37°C) and oscillation (200 rpm) to determine the release of mānuka oil and β-triketones. At each time interval (0, 1, 3, 5, 7, 10, and 14 days), the samples were centrifuged at 13,000 g for 5 min and 1 ml of the supernatant collected and measured spectrophotometrically at 280 nm (n = 3). PBS (100 ml) was left with the collected particles at the bottom of the tube after centrifugation and fresh PBS (1 ml) added for the next timepoint. Supernatant collected from the unloaded CNPs was used as a blank. The concentration of the mānuka oil, and β-triketones was calculated through interpolation from a standard curve ranging from 316.4 to 1000 nl/ml for mānuka oil and 42.2-562 nl/ml for β-triketones. The total release was calculated and then percentages at each time point derived.

2.5. Antimicrobial activity assessed by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC and MBC of mānuka oil and β-triketones loaded CNPs, along with the unloaded nanoparticles were measured for *E. faecalis* V583, *S. mutans* UA159, *P. gingivalis* ATCC33277, and *S. aureus* ATCC6538 according to Kowalska-Krochmal et al. [21] with some modifications. The highest concentration of the nanoparticles tested was 40 mg/ml with technical replication n = 3.

2.6. Metabolic activity of human Mesenchymal Stem Cells (MSCs)

MSCs were amplified in MesenPRO RS™ basal medium (#12747-010, Gibco, MT, USA) supplemented with MesenPRO RS™ Growth supplement (#12748-018, Gibco, MT, USA), GlutaMAX™ (100x; #35050-061, Gibco, MT, USA), antibiotic-antimycotic reagent (100x; #15240-062, Gibco, MT, USA) and gentamicin reagent (#15710-064, Gibco, MT, USA) making MesenPRO complete medium.

Cells were seeded at 3000 cells/cm^2 in 96-well plates in 50% of MesenPRO RS™ complete medium and 50% DMEM media (#30-2002, ATCC, VA, USA) which had been supplemented with 10% FBS (#10091-148, Gibco, MT, USA), antibiotic-antimycotic reagent, and gentamicin reagent.

Nanoparticles were UV sterilized for 30 min in the hood and dissolved in the (MesenPRO/DMEM) at concentrations 100, 75, 50, 25, 12.5, 6.25. 3.125, 1.56 mg/ml (100 ml/well) then incubated for 20 h at 37°C.

PrestoBlue® cell viability reagent (# A13261, Life Technologies, Oregon, USA) was added (10 ml/well) and incubated for 4 h for the 24-h time point. PrestoBlue® was repeated at 48 and 72 h. Fluorescence was measured at an excitation of 560 nm and an emission of 590 nm on a Synergy™ 2 Multi-mode microplate reader (Biotek, USA). The experiment was independently repeated 3 times. Two-Way ANOVA was used to compare each group with the control cells and Dunnett post-hoc test conducted using GraphPad Prism (version 9.4.1), with the mean, and standard deviation plotted. Statistical significance was calculated at $p < 0.05$.

2.7. Cell staining

Cells were fixed at 72 h with 100 µl of 10% formalin in each well for 15 min and stained with 200 µl of crystal violet (#C0775-25G, Sigma-Aldrich®, St Louis, USA) for 15 min. Finally, the wells were washed with 200 µl of PBS and then air dried for 15 min. Cells were visualized using EVOS M5000 Imaging System.

2.8. Validation of the effect of trehalose used as cryoprotectant on human MSCs

This experiment was conducted to investigate the possible effects of the trehalose within the freeze-dried material on the metabolic activity of MSCs. The mass of the solid components of chitosan, TPP, and trehalose were summed to give a final percentage in the freeze-dried nanoparticles. To control for the effect of trehalose, its concentration was standardized across treatments. The 100 mg/ml particle suspension contained 66.22 mg of trehalose per 100 mg of nanoparticles. Accordingly, the 12.5 mg nanoparticle samples (containing 8.27 mg of intrinsic trehalose) were supplemented with an additional 57.95 mg of trehalose to match the total trehalose content present in the 100 mg/ml condition. The metabolic activity of MSCs was measured with PrestoBlue® cell viability reagent as described previously using 12.5 mg/ml of chitosan, chitosan/mānuka oil or chitosan/β-triketones nanoparticles with and without the 66.22% trehalose.

3. Results

3.1. Production of nanoparticles

Gas chromatographic analysis of the mānuka oil and β-triketone extracts (Supplementary Table 1) showed total β-triketones content (Flavesone, Leptospermone, Iso-Leptospermone, and Grandiflorone) in the mānuka oil extract of 29.2% and the β-triketone extract of 99.7%.

CNPs were prepared with different formulations summarized in Table 1, where three different ratios of chitosan:TPP were investigated and two different pH settings for both chitosan (pH: 3.9, 5.5) and TPP (pH: 9.0, 5.5) [18]. The average PDI was the lowest in sample 4 for chitosan alone nanoparticles (0.654), sample 16 for CNPs with mānuka oil (0.471), and sample 28 for chitosan with β-triketone nanoparticles (0.379). These three samples all had the same chitosan:TPP ratio of 5:1 with chitosan pH 5.5 and TPP pH 9. Other potential useful formulations with similar or slightly higher PDIs were found with a ratio 3:1 with chitosan pH 5.5 and TPP pH 5.5 (samples 12, 24, and 36). DLS peaks are presented for these six formulations and show a single peak with the chitosan:TPP ratio of 5:1 with chitosan pH 5.5 and TPP pH 9 making it the preferred option (Fig. 1). The visual appearance of this formulation when stained with Lilli oil red O confirmed less aggregation and more uniformity in size distribution of the nanoparticles (Fig. 2). The lower the chitosan to TPP ratio (i.e. 4:1 and 3:1) the more aggregation of the chitosan particles was noted (Table 1). Similarly with unadjusted chitosan pH (3.9) compared to the one adjusted to pH 5.5. Therefore, samples 4, 16, and 28 with a chitosan:TPP ratio of 5:1, with chitosan adjusted to pH 5.5 and TPP unadjusted at pH 9, was deemed the optimum formulation for producing nanoparticles.

Unloaded CNPs size ranged from 279 to 550 nm, mānuka oil nanoparticles from 237 to 944 nm and β-triketones nanoparticles from 168 to 573. Larger particles were associated with the 3:1 ratio, aggregate formation, and pH 5.5 in both solutions (Table 1). The sizes for the selected chitosan, chitosan/mānuka oil and β-triketone nanoparticle formulations were similar at 327, 337, and 288 nm respectively. Interestingly, in all except two formulations with chitosan/β-triketone their nanoparticles were smallest compared to the chitosan alone and mānuka oil nanoparticles. The zeta potential for unloaded CNPs was +23 mV, while chitosan/mānuka oil measured +27 mV and chitosan/β-triketones nanoparticles +28 mV.

The yield after freeze-drying was consistently much higher for chitosan/mānuka oil (297-369 mg) and chitosan/β-triketones nanoparticles (333-465 mg) compared to chitosan alone (225-276 mg). In the selected samples the yield was 266 mg for chitosan, 338 mg for chitosan/mānuka oil, and 370 mg chitosan/β-triketones nanoparticles, following the trend seen for all formulations in Table 1.

SEM images of the CNPs confirmed the structure and uniformity of the formulated nanoparticles (Fig. 3).

The encapsulation efficiency was higher in the selected samples compared to the other formulations; however, we noticed slight variations with different batches. The encapsulation efficiency for mānuka oil sample was 89.2% and for β-triketones 89.5%.

3.2. In vitro release of mānuka oil and β-triketones

Release of mānuka oil and β-triketones from CNPs are shown in Fig. 4, with an immediate burst release observed upon reconstitution of the freeze-dried nanoparticles in PBS and after one day, then moderate release over 5 days for mānuka oil and 7 days for β-triketones. A low release followed until day 14.

3.3. Antimicrobial activity

MIC and MBC data for the nanoparticles are summarized in Table 2. CNPs had both MIC and MBC values above the tested concentration of 40 mg/ml, except with *E. faecalis* where the MIC was at 40 mg/ml

Table 1
Comparison of the different formulations of chitosan nanoparticles (NP) with different tripolyphosphate ratios and pH solutions. Optimal formulations are shown in bold.

Sample no.	NP type	Visual appearance	Mass: mass ratio Ch: TPP	Chitosan pH	TPP pH	TPP (final concentration) %	Avg. particle size (nm)	PDI	Freeze drying yield (mg)	Enc. Efficiency %
1	Ch	X	5:1	3.9	9	0.050	366	0.781	241	---
2	Ch	X	4:1	3.9	9	0.062	297	0.810	229	---
3	Ch	X	3:1	3.9	9	0.083	321	0.869	242	---
4	**Ch**	**X**	**5:1**	**5.5**	**9**	**0.050**	**327**	**0.654**	**266**	**---**
5	Ch	Ö	4:1	5.5	9	0.062	377	0.822	259	---
6	Ch	¯	3:1	5.5	9	0.083	487	0.906	225	---
7	Ch	X	5:1	3.9	5.5	0.050	279	0.940	240	---
8	Ch	X	4:1	3.9	5.5	0.062	336	0.886	245	---
9	Ch	Ö	3:1	3.9	5.5	0.083	416	0.835	242	---
10	Ch	Ö	5:1	5.5	5.5	0.050	400	0.911	267	---
11	Ch	ÖÖ¯	4:1	5.5	5.5	0.062	551	0.812	276	---
12	**Ch**	**ÖÖÖ¯**	**3:1**	**5.5**	**5.5**	**0.083**	**550**	**0.769**	**268**	**---**
13	Ch/MO	Ö	5:1	3.9	9	0.050	356	0.607	307	81.89
14	Ch/MO	Ö	4:1	3.9	9	0.062	269	0.668	323	82.10
15	Ch/MO	Ö	3:1	3.9	9	0.083	282	0.700	317	82.60
16	**Ch/MO**	**Ö**	**5:1**	**5.5**	**9**	**0.050**	**336**	**0.471**	**338**	**89.21**
17	Ch/MO	Ö	4:1	5.5	9	0.062	378	0.555	335	70.08
18	Ch/MO	Ö	3:1	5.5	9	0.083	944	0.908	355	67.50
19	Ch/MO	Ö	5:1	3.9	5.5	0.050	237	0.838	312	88.90
20	Ch/MO	Ö	4:1	3.9	5.5	0.062	292	0.878	297	82.01
21	Ch/MO	Ö	3:1	3.9	5.5	0.083	465	0.905	317	83.20
22	Ch/MO	Ö	5:1	5.5	5.5	0.050	546	0.512	344	66.80
23	Ch/MO	ÖÖ	4:1	5.5	5.5	0.062	450	0.560	369	70.09
24	**Ch/MO**	**ÖÖÖ**	**3:1**	**5.5**	**5.5**	**0.083**	**439**	**0.468**	**344**	**75.90**
25	Ch/TK	ÖÖ	5:1	3.9	9	0.050	573	0.715	444	69.65
26	Ch/TK	ÖÖ	4:1	3.9	9	0.062	204	0.593	389	75.35
27	Ch/TK	ÖÖ	3:1	3.9	9	0.083	257	0.506	372	70.03
28	**Ch/TK**	**Ö**	**5:1**	**5.5**	**9**	**0.050**	**288**	**0.379**	**370**	**89.50**
29	Ch/TK	Ö	4:1	5.5	9	0.062	322	0.418	405	27.90
30	Ch/TK	Ö	3:1	5.5	9	0.083	386	0.523	404	37.69
31	Ch/TK	ÖÖ	5:1	3.9	5.5	0.050	186	0.619	347	67.60
32	Ch/TK	ÖÖ	4:1	3.9	5.5	0.062	240	0.685	333	74.30
33	Ch/TK	ÖÖ	3:1	3.9	5.5	0.083	415	0.613	553w	72.02
34	Ch/TK	ÖÖ	5:1	5.5	5.5	0.050	424	0.514	444	64.95
35	Ch/TK	ÖÖ	4:1	5.5	5.5	0.062	409	0.488	465	48.19
36	**Ch/TK**	**ÖÖ**	**3:1**	**5.5**	**5.5**	**0.083**	**322**	**0.603**	**465**	**55.40**

X = Clear solution, Ö = Opalescent solution, ¯ = Aggregates; Ch: Chitosan nanoparticles; Ch/MO: Chitosan/mānuka oil nanoparticles; Ch/TK: Chitosan/β-triketones nanoparticles; TPP: Sodium tripolyphosphate; PDI: Polydispersity index; Enc.: Encapsulation; w: wet.

β-triketone encapsulated nanoparticles showed consistently better MIC values than the mānuka oil nanoparticles.

3.4. Metabolic activity of human mesenchymal stem cells

The metabolic activity of MSCs (Fig. 5a–c) in the three nanoparticle groups (chitosan alone, chitosan/mānuka oil, and chitosan/β-triketones) at low concentration (1.56 mg/ml and 3.125 mg/ml) was above the control at the three time points tested. The three groups demonstrated similar trends with decreased metabolic activity at 12.5 mg/ml and 25 mg/ml. Interestingly, concentrations from 50 to 100 mg/ml then showed increased metabolic activity for all groups. When the three groups were compared at the 72 h timepoint a similar dose pattern was found (Fig. 5d). This result was confirmed when cells

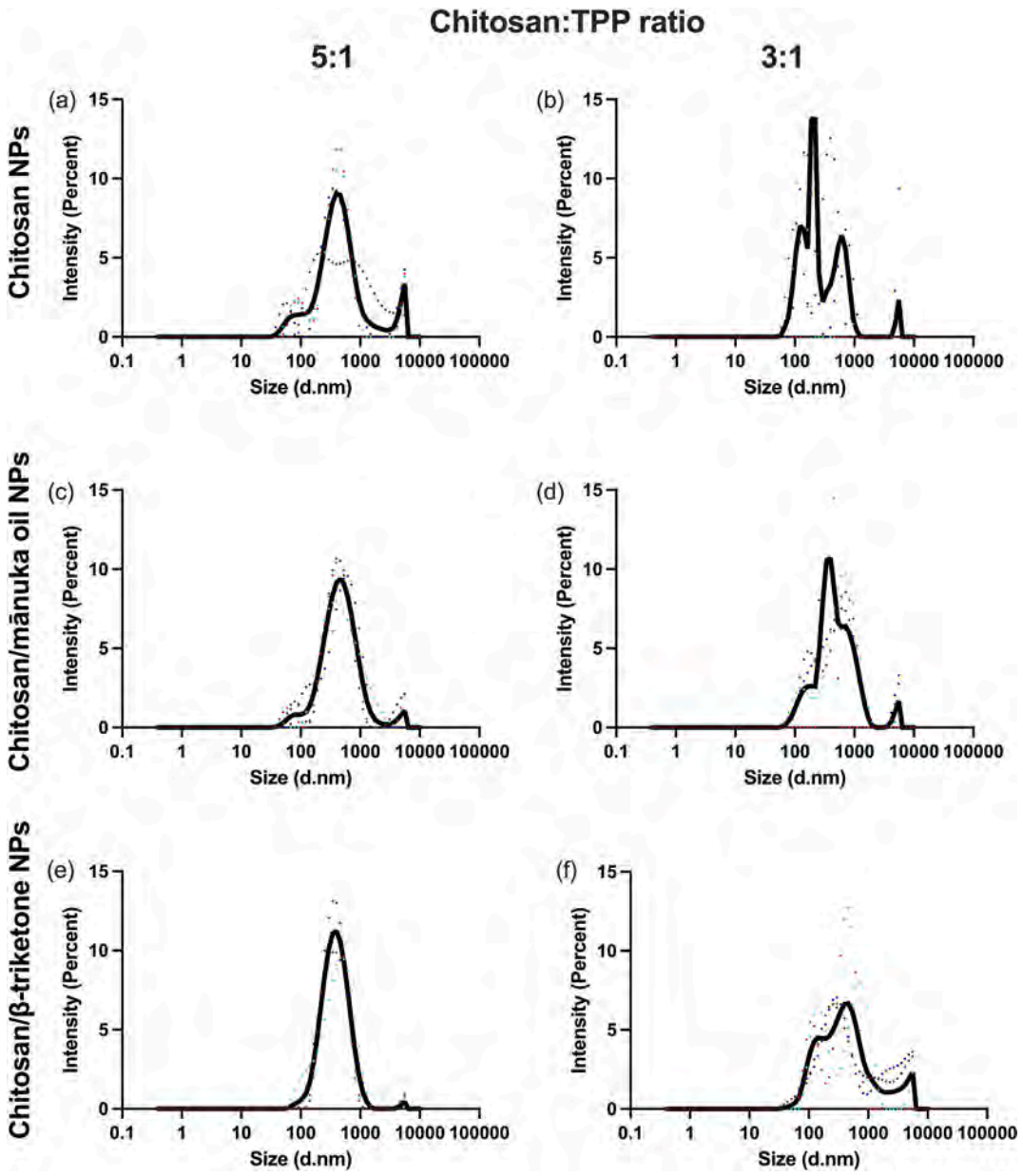


Fig. 1. Dynamic light scattering analysis of selective formulations of unloaded (a, b) and loaded chitosan (c-f) nanoparticles (NPs) (average of 4 runs with replicates shown as colored dots). Chitosan was at pH 5.5 for all formulations. Nanoparticles were produced with chitosan:TPP ratios of 5:1 and TPP at pH 9 (left) or ratios of 3:1 and TPP at pH 5.5 (right). Sample numbers (SN) refer to Table 1: (a) SN 4: Chitosan:TPP ratio 5:1, Chitosan pH 5.5, TPP pH 9. (b) SN 12: Chitosan:TPP ratio 3:1, Chitosan pH 5.5, TPP pH 5.5. (c) SN 16: Chitosan:TPP ratio 5:1, Chitosan pH 5.5, TPP pH 9. (d) SN 24: Chitosan:TPP ratio 3:1, Chitosan pH 5.5, TPP pH 5.5.(e) SN 28: Chitosan:TPP ratio 5:1, Chitosan pH 5.5, TPP pH 9. (f) SN 36: Chitosan:TPP ratio 3:1, Chitosan pH 5.5, TPP pH 5.5.

were stained with crystal violet after 72 h of incubation (Fig. 6). A distinctive morphological difference was visible for the cells with high nanoparticle concentrations with cells more spindle shaped with longer processes.

No change in the metabolic activity of MSCs was observed when complementing the nanoparticles with 12.5 mg/ml of trehalose as the level expected to be present at 100 mg/ml (Fig. 5e). The metabolic activity of the cells remained very low (less than 20%) compared to the control.

4. Discussion

Capturing the traditional benefits of natural antimicrobials requires innovative delivery mechanisms that maintain bioactivity and substantivity for longer periods. Oils such as mānuka with its antimicrobial effect, are hydrophobic and volatile in nature, and as such are challenging to deliver effectively. Unlike the traditional methods adopting oil emulsions, this study has provided an effective method of not only encapsulating mānuka oil but also its most prominent active ingredient of β-triketones. The produced nanoparticles could be used alone or as an adjunct to graft materials, surgical membranes, gels or wafers where antimicrobial benefits might be beneficial.

CNPs are currently one of the most promising biobased polymeric particles [22]. They provide many advantages in a naturally based drug delivery system including mucoadhesion, encapsulation of a variety of payloads and a tailorable release profile [23]. Despite the various methods described for producing the CNPs [16], it is challenging to produced nanoparticles with high uniformity and encapsulation efficiency. Ionic gelation of chitosan using TPP has been widely reported, however the size and distribution of the particles are affected by

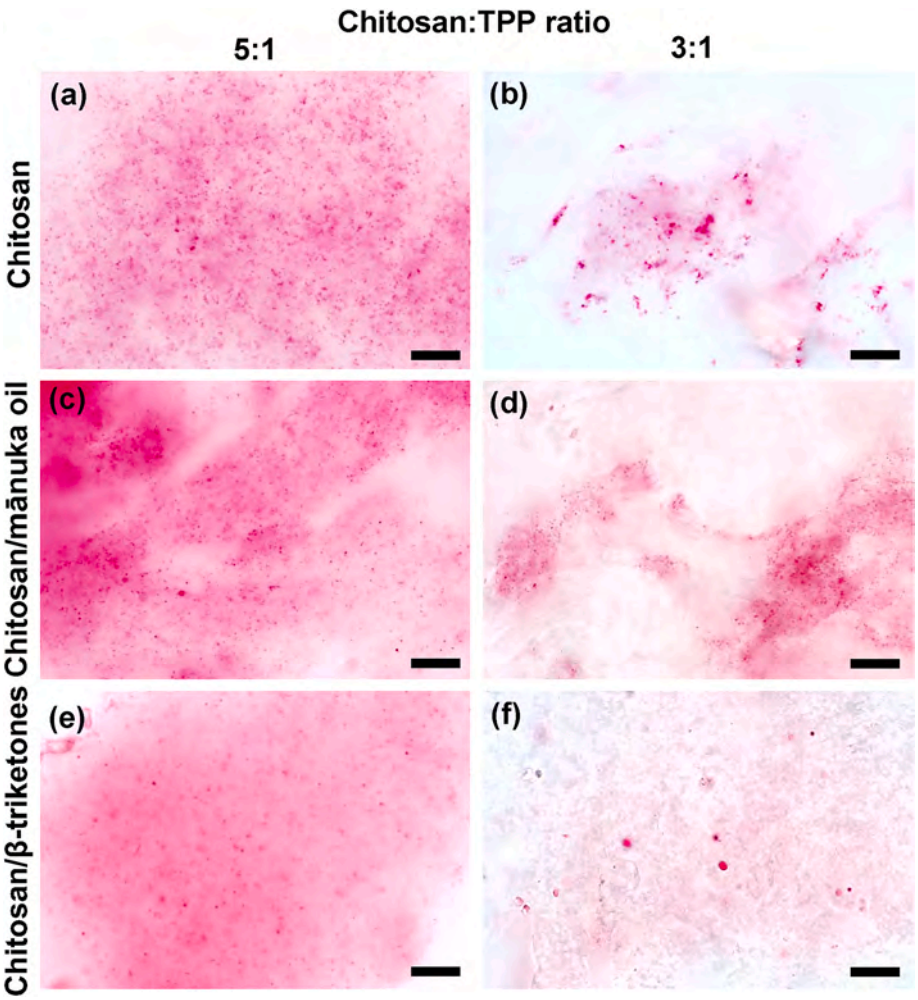


Fig. 2. Representative microscopy of unloaded (a, b) and loaded chitosan (c-f) nanoparticles stained with Lilli oil red O. Chitosan was at pH 5.5 for all formulations. Nanoparticles were produced with chitosan:TPP ratios of 5:1 and TPP at pH 9 (left) or ratios of 3:1 and TPP at pH 5.5 (right). Sample numbers (SN) refer to Table 1: (a) SN 4: Chitosan:TPP ratio 5:1, Chitosan pH 5.5, TPP pH 9. (b) SN 12: Chitosan:TPP ratio 3:1, Chitosan pH 5.5, TPP pH 5.5. (c) SN 16: Chitosan:TPP ratio 5:1, Chitosan pH 5.5, TPP pH 9. (d) SN 24: Chitosan:TPP ratio 3:1, Chitosan pH 5.5, TPP pH 5.5.(e) SN 28: Chitosan:TPP ratio 5:1, Chitosan pH 5.5, TPP pH 9. (f) SN 36: Chitosan:TPP ratio 3:1, Chitosan pH 5.5, TPP pH 5.5. Scale bars 10 mm.

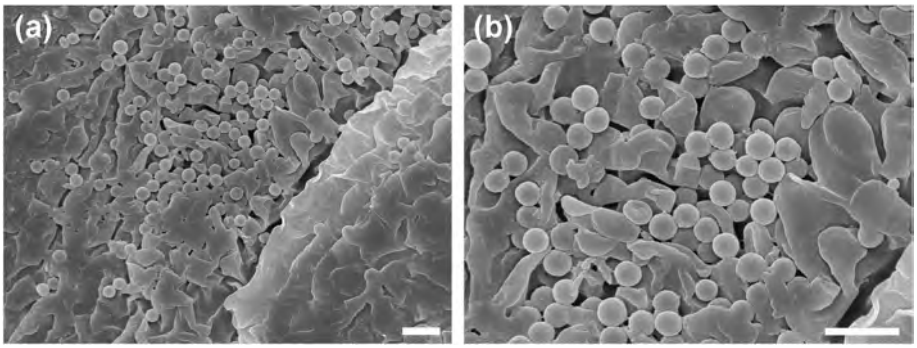


Fig. 3. Scanning electron microscopic images of chitosan nanoparticles within trehalose. Multiple spheres of <400 nm are evident. Scale bars = 1 μm.

multiple factors including the pH of the chitosan and TPP solutions, the molecular weight of chitosan utilized, as well as the chitosan:TPP ratios [24–26].

Optimization of nanoparticle formation used three different chitosan:TPP mass ratios (5:1, 4:1, and 3:1), and two different pH values for each solution (3.9 "unadjusted" and 5.5 for chitosan, and 9 "unadjusted" and 5.5 for TPP). The higher the chitosan to TPP ratio (5:1) the smaller the particle size produced with less tendency toward aggregation. Adjusting the pH of the chitosan solution to 5.5 before adding the TPP provided a more optimal PDI than when using the more acidic unadjusted solution. Lowering the pH of the TPP solution from its initial alkaline pH 9 to 5.5 together with a chitosan:TPP ratio of 3:1 resulted in a comparable PDI to the one obtained with the adjusted chitosan solution (pH 5.5), however the particle size was much bigger and a visible aggregation was noticed in the resultant solution. Similar results were obtained when either mānuka oil or β-triketones were added to the

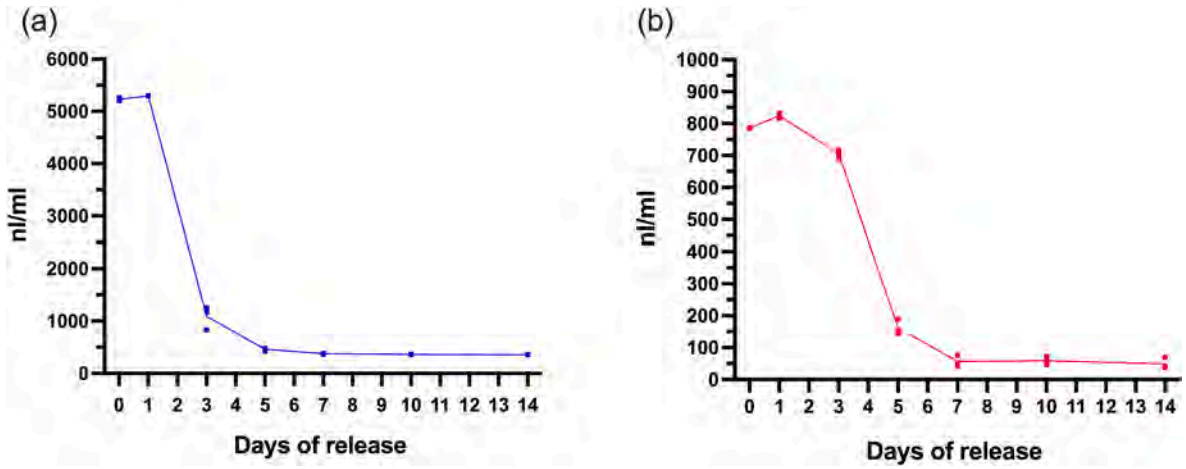


Fig. 4. Release of (a) mānuka oil and (b) β-triketones from chitosan nanoparticles in PBS over a period of 14 days. Mean, n = 3.

Table 2
MIC and MBC values of nanoparticles (NP) of chitosan alone, chitosan/mānuka oil, and chitosan/β-triketones against four bacteria (n = 3).

Bacteria	MIC			MBC		
	Chitosan-NP (mg/ml)	MO-NP (mg/ml)	TK-NP (mg/ml)	Chitosan-NP (mg/ml)	MO-NP (mg/ml)	TK-NP (mg/ml)
E. faecalis	40	6.66	0.62	>40	>40	>40
S. mutans	>40	20	10	>40	>40	40
S. aureus	>40	20	5	>40	20	20
P. gingivalis	>40	20	5	>40	40	10

chitosan solution. These results agreed with Rampino et al. [18] in which the 5:1 chitosan:TPP mass ratio resulted in the best PDI compared to the other ratios investigated. Others who have incorporated essential oils into chitosan either do no report a PDI [27–29], or report values similar to ours, for the optimized production, of 0.471 for mānuka oil and 0.379 for β-triketones chitosan nanospheres: *Ocimum basilicum* PDI = 0.222-0.425 [30], *Cymbopogon commutatus* and *Carvacrol* and PDI = 0.3-0.5 [31,32], *Lavandula dentata* PDI = 0.57 ± 0.03 [33], saffron PDI = 0.498 [34] and oregano and thyme PDI = 0.42-0.53 [35]. Given the intraoral applications it would be ideal to improve the uniformity of the nanoparticles with a microfluidics approach.

The zeta potential was used to detect the magnitude of the surface charge, with CNPs having a value of +23 mV which increased with to +27 mV and +28 mV with mānuka oil or β-triketone inclusion. The positive charge can be attributed to the amino groups of the chitosan. The charge change on incorporation of an essential oil is strongly associated with the charge of the molecules [32], which in this case was close to neutral. In general, nanoparticles with zeta potential values > +30 mV indicate high stability (preventing aggregation), around +20 mV show a short-term stability, and values < +5 mV represent low stability (fast aggregation) [31].

A critical step in the production of nanoparticles was the addition of a cryoprotectant during the freeze-drying. In this study we utilized trehalose at 5% w/v in accordance with Mohammady et al. [36]. Trehalose is non-nutritional and did not affect the antimicrobial properties, as was present in all formulations including the chitosan alone nanoparticles with MBC >40, nor affecting the metabolic activity of the MSCs.

The release profile showed release of mānuka oil and β-triketones for almost a week. β-triketone CNPs did however maintain high release for three days compared to the one day for mānuka oil. Tailored release from the nanoparticles also be achieved by incorporating them into other bioengineered products for delivery.

In this study, four different bacterial species were selected providing a range of susceptibility to the nanoparticles. *E. faecalis* bacterium is considered one of the most common nosocomial infections and have a high emerging resistance to antibiotics [37]. It is very common bacterium in failed root canal treatment [38]. *S. mutans* has been implicated as a primary causative agent of dental caries [39]. *P. gingivalis* is mainly involved in the progression of periodontal diseases [40], it has also implications in the onset of rheumatoid arthritis, cardiovascular pathologies, and neurodegenerative pathologies [41]. *S. aureus* is involved in skin infections resulting in boils and blisters [42]. This selection of species was primarily to include Gram-positive, Gram-negative, aerobic and anaerobic bacteria and allow scope for expanded applications to other clinical applications.

The antibacterial properties of mānuka oil have previously been reported [43], and the activity attributed to its β-triketone content. In this study we encapsulated both the commercially available mānuka oil and the purified β-triketones in CNPs and found higher activity with of β-triketones nanoparticles [44]. A difference in MIC and MBC values was observed and this was dependent on bacterial species type. *E. faecalis* showed a low MIC but a high MBC probably due to its intrinsic tolerance to drugs targeting the cell wall. The loading of the nanoparticles required in a clinical setting will need to be confirmed with reference to treatment modality and target requirements with biofilms presenting a higher challenge. We have calculated the percentage of active in 40 mg/ml of loaded nanoparticles taking into consideration the trehalose and this equates to a 0.336% (v/v) solution of active, consistent with known MIC/MBCs of mānuka oil on a range of oral bacteria [45,46]. Chitosan alone has been reported to have some antibacterial properties [47], and in this study *E. faecalis* had an MIC of 40 with all other species >40.

The metabolic activity of the MSCs in this study indicates that the effect of CNPs changes with the form and quantity of the nanoparticles. Moderate concentrations of nanoparticles drastically reduced the metabolic activity of the cells whether incorporating mānuka oil, β-triketones or chitosan alone, and these adverse results were partially reversed by increasing the concentration of the nanoparticles up to 100 mg/ml. This response was supported by the staining of cells at 72 h showing a biphasic dose response. This was in agreement with Jesus et al. [48] where the CNPs were found to be more cytotoxic than the corresponding polymer. The partially restored metabolic activity with the high concentration (100 mg/ml) of nanoparticles was not due to the added cryoprotectant (trehalose) and may be attributed the interaction with the nanoparticles themselves or to issues with the assay itself. Chitosan is known to have beneficial effects in MCSs, modulating critical Wnt/β-catenin, Notch, and HIF-1α signaling which enhances survival,

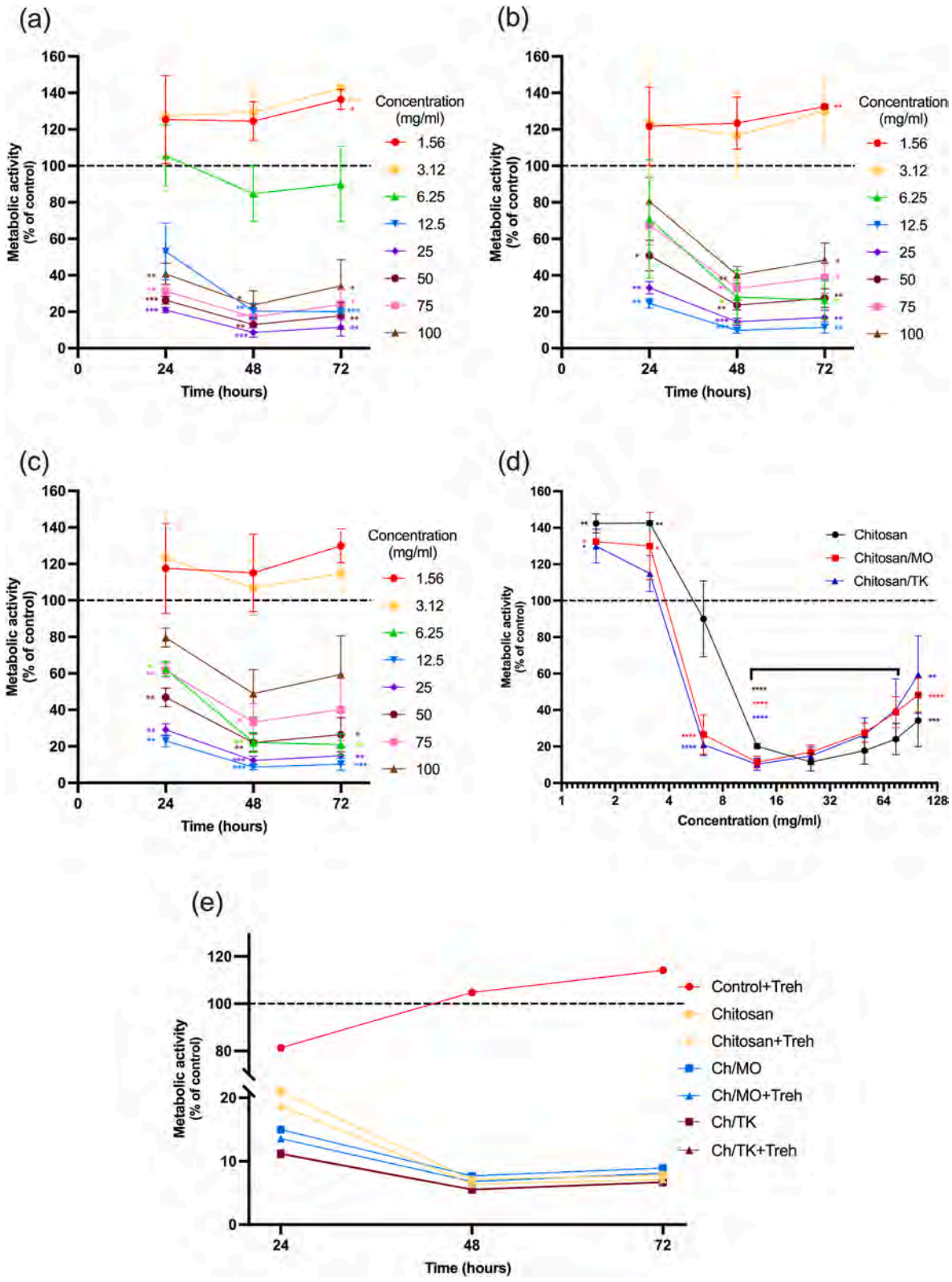


Fig. 5. Metabolic activity of human Mesenchymal Stem Cells (MSCs) with different concentrations of (a) chitosan nanoparticles, (b) chitosan/mānuka oil nanoparticles, (c) chitosan/β-triketones nanoparticles. (d) Comparison of the three groups at (72-h) time point. (e) Trehalose neutral effect on the metabolic activity of MSCs at a nanoparticle concentration of 12.5 mg/ml (n = 3). MO = mānuka oil; Ch = Chitosan; TK = β-triketones; Treh = trehalose. Mean ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$. Dotted line = control.

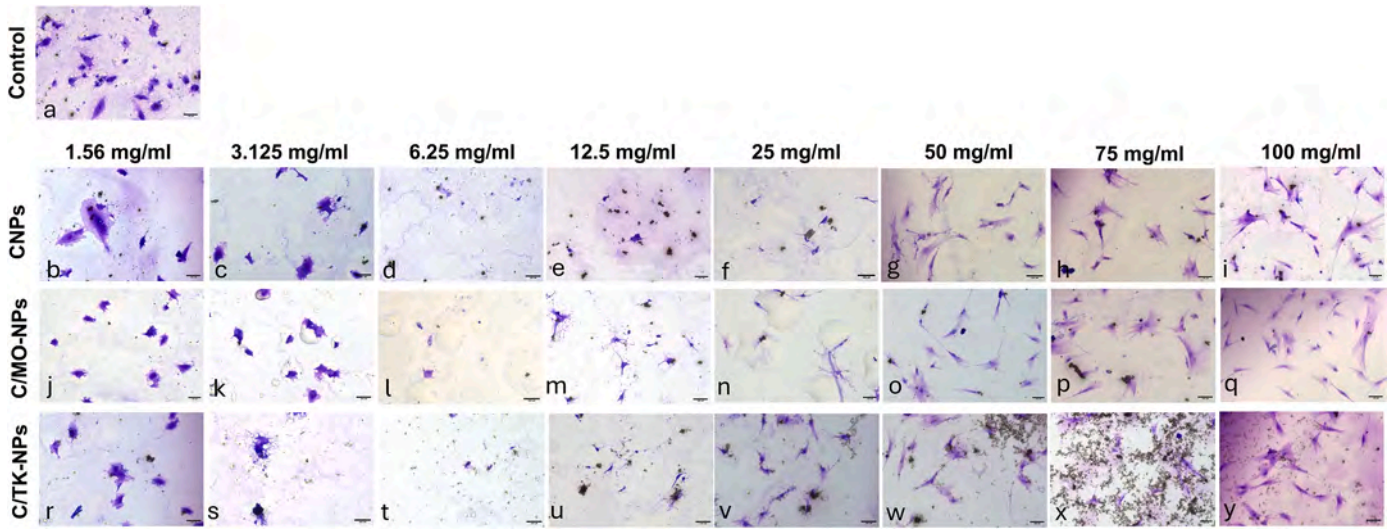


Fig. 6. Mesenchymal stem cells stained with crystal violet after 72 h incubation with (a) control containing no nanoparticles, (b-i) chitosan nanoparticles (CNPs), (j-q) chitosan/mānuka oil nanoparticles (C/MO-NPs) and (r-y) chitosan/β-triketone nanoparticles (C/TK-NPs), at different concentrations. Scale bar 100 mm.

self-renewal, osteogenic differentiation and immunoregulatory properties [49]. Further mechanistic studies on the MSC function are recommended to fully understand this phenomenon. Interestingly, addition of actives to the CNPs did not dramatically affect metabolic activity of the cells. In this research cytotoxicity was tested up to 100 mg/ml to reflect the MIC/MBC values which indicated an antimicrobial dose of 20 mg/ml to reach the MIC and higher for the MBC. The *in vitro* models t*in vitro* an imperfect reflection of the *in vivo* situation where the antimicrobial activity and cytotoxicity are affected not only by dose but substantivity, adhesion, release, mechanical forces and salivary flow. Further research is being conducted on gel and matrix carries for the nanoparticles which can also modulate their *in vivo* activity.

The distinctive phenotypical changes of MSCs including flattening and enlarged cell morphology manifested with the high concentration of nanoparticles could be a sign of cellular senescence [50], which could potentially lead to decline in their differentiation and proliferation ability. However, further studies will be required to understand the exact reason for those changes and whether nanoparticles would have similar effect when incorporated into a bioengineered product.

Conclusion

Mānuka oil and most notably β-triketones encapsulated in CNPs provide novel antimicrobial systems that could be incorporated into hydrogels, membranes, graft materials or wafers to produce bioengineered products for the oral cavity.

Ethical approval

No ethical approvals were required for this research.

Author contribution

Amira S Salem, Study design, data collection, data analysis, writing, Warwick J. Duncan, Study design, funding, data interpretation, Nerida Downes, Study design, data analysis, Dina Abdelmoneim, Data collection, data interpretation, data analysis, Gemma C. Porter, Funding, data interpretation, Natalie J. Medlicott, Data collection, funding, data interpretation, Ghsaq Alhamdani, Data colleciton, data analysis, Chen Chen, Data colleciton, data analysis, Dawn E Coates, Study design, data collection, writing, funding, data analysis, data interpretation.

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Declaration of competing interest

The authors declare no conflicts of interest regarding this manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.job.2026.100828.

References

[1] Mathur S, Hoskins C. Drug development: lessons from nature. Biomed Rep 2017;6:612–4.
[2] Ventola CL. The antibiotic resistance crisis: part 1: causes and threats. P T 2015;40:277–83.
[3] Garcia-Salinas S, Elizondo-Castillo H, Arruebo M, Mendoza G, Irusta S. Evaluation of the antimicrobial activity and cytotoxicity of different components of natural origin present in essential oils. Molecules 2018;23.
[4] Patel RN. Wood anatomy of the dicotyledons indigenous to New Zealand. N Z J Bot 1973;11:421–34.
[5] Salmon JT. The native trees of New Zealand/J. T Salmon Wellington: Reed 1980.
[6] Lis-Balchin M, Hart SL, Deans SG. Pharmacological and antimicrobial studies on different tea-tree oils (Melaleuca alternifolia, Leptospermum scoparium or Manuka and Kunzea ericoides or Kanuka), originating in Australia and New Zealand. Phytother Res 2000;14:623–9.
[7] Porter NG, Wilkins AL. Chemical, physical and antimicrobial properties of essential oils of Leptospermum scoparium and Kunzea ericoides. Phytochemistry 1999;50:407–15.
[8] Porter GC, Safii SH, Medlicott NJ, Duncan WJ, Tompkins GR, Coates DE. Formulation of a semisolid emulsion containing Leptospermum scoparium essential oil and evaluation of In vitro antimicrobial and Antibiofilm Efficacy. Planta Med 2021;87:253–66.
[9] Miastkowska M, Michalczyk A, Figacz K, Sikora E. Nanoformulations as a modern form of biofungicide. J Environ Health Sci Eng 2020;18:119–28.

[10] Vervelle A, Mouhyi J, Del Corso M, Hippolyte MP, Sammartino G, Dohan Ehrenfest DM. [Mouthwash solutions with microencapsuled natural extracts: efficiency for dental plaque and gingivitis]. Rev Stomatol Chir Maxillofac 2010; 111:148–51.
[11] Aranaz I, Alcantara AR, Civera MC, Arias C, Elorza B, Heras Caballero A, Acosta N. Chitosan: an overview of its properties and applications. Polymers 2021;13.
[12] Szulc M, Lewandowska K. Biomaterials based on Chitosan and its derivatives and their potential in tissue engineering and other biomedical Applications-A review. Molecules 2022;28.
[13] Shariatinia Z. Pharmaceutical applications of chitosan. Adv Colloid Interface Sci 2019;263:131–94.
[14] Bashir SM, Ahmed Rather G, Patricio A, Haq Z, Sheikh AA, Shah M, Singh H, Khan AA, Imtiyaz S, Ahmad SB, Nabi S, Rakhshan R, Hassan S, Fonte P. Chitosan nanoparticles: a versatile platform for biomedical applications. Materials 2022;15.
[15] Singla AK, Chawla M. Chitosan: some pharmaceutical and biological aspects–an update. J Pharm Pharmacol 2001;53:1047–67.
[16] Yanat M, Schroën K. Preparation methods and applications of chitosan nanoparticles; with an outlook toward reinforcement of biodegradable packaging. React Funct Polym 2021;161:104849.
[17] Ahmed TA, Aljaeid BM. Preparation, characterization, and potential application of chitosan, chitosan derivatives, and chitosan metal nanoparticles in pharmaceutical drug delivery. Drug Des Devel Ther 2016;10:483–507.
[18] Rampino A, Borgogna M, Blasi P, Bellich B, Cesaro A. Chitosan nanoparticles: preparation, size evolution and stability. Int J Pharm 2013;455:219–28.
[19] Ma S, Adayi A, Liu Z, Li M, Wu M, Xiao L, Sun Y, Cai Q, Yang X, Zhang X, Gao P. Asymmetric Collagen/chitosan membrane containing minocycline-loaded Chitosan nanoparticles for guided bone regeneration. Sci Rep 2016;6:31822.
[20] Othman N, Masarudin MJ, Kuen CY, Dasuan NA, Abdullah LC, Md Jamil SNA. Synthesis and optimization of Chitosan nanoparticles loaded with L-Ascorbic acid and thymoquinone. Nanomaterials 2018;8.
[21] Kowalska-Krochmal B, Dudek-Wicher R. The Minimum inhibitory concentration of antibiotics: methods, interpretation. Clinical Relevance Pathogens 2021;10.
[22] Baharlouei P, Rahman A. Chitin and chitosan: prospective biomedical applications in drug delivery, cancer treatment, and wound healing. Mar Drugs 2022;20.
[23] Yee Kuen C, Masarudin MJ. Chitosan nanoparticle-based System: a new Insight into the promising controlled release System for lung cancer treatment. Molecules 2022;27.
[24] Algharib SA, Dawood A, Zhou KX, Chen DM, Li C, Meng KY, Zhang AX, Luo WH, Ahmed S, Huang LL, Xie SY. Preparation of chitosan nanoparticles by ionotropic gelation technique: effects of formulation parameters and in vitro characterization. J Mol Struct 2022;1252.
[25] Bhumkar DR, Pokharkar VB. Studies on effect of pH on cross-linking of chitosan with sodium tripolyphosphate: a technical note. AAPS PharmSciTech 2006;7:E50.
[26] Mattu C, Li R, Ciardelli G. Chitosan nanoparticles as therapeutic protein nanocarriers: the effect of ph on particle formation and encapsulation efficiency. Polym Composite 2013;34:1538–45.
[27] Hosseini SF, Zandi M, Rezaei M, Farahmandghavi F. Two-step method for encapsulation of oregano essential oil in chitosan nanoparticles: preparation, characterization and in vitro release study. Carbohydr Polym 2013;95:50–6.
[28] Song X, Wang L, Liu T, Liu Y, Wu X, Liu L. Mandarin (Citrus reticulata L.) essential oil incorporated into chitosan nanoparticles: characterization, anti-biofilm properties and application in pork preservation. Int J Biol Macromol 2021;185: 620–8.
[29] Sotelo-Boyás M, Correa-Pacheco Z, Bautista-Baños S, Gómez y Gómez Y. Release study and inhibitory activity of thyme essential oil-loaded chitosan nanoparticles and nanocapsules against foodborne bacteria. Int J Biol Macromol 2017;103: 409–14.
[30] Cai M, Wang Y, Wang R, Li M, Zhang W, Yu J, Hua R. Antibacterial and antibiofilm activities of chitosan nanoparticles loaded with Ocimum basilicum L. essential oil. Int J Biol Macromol 2022;202:122–9.
[31] Soltanzadeh M, Peighambardoust SH, Ghanbarzadeh B, Mohammadi M, Lorenzo JM. Chitosan nanoparticles encapsulating lemongrass (Cymbopogon commutatus) essential oil: physicochemical, structural, antimicrobial and in-vitro release properties. Int J Biol Macromol 2021;192:1084–97.
[32] Mondéjar-López M, López-Jimenez AJ, García Martínez JC, Ahrazem O, Gómez-Gómez L, Niza E. Comparative evaluation of carvacrol and eugenol chitosan nanoparticles as eco-friendly preservative agents in cosmetics. Int J Biol Macromol 2022;206:288–97.
[33] Bourenane EK, Amokrane D, Mohammedi A, Bouras D, Wu X, Andreasen C, Khezami L, Fellah M. A new chitosan-based nanoformulation of Lavandula dentata essential oil for sustainable control of green Citrus Aphids. ChemistrySelect 2026; 11.
[34] Astutiningsih F, Anggrahini S, Fitriani A, Supriyadi S. Optimization of Saffron essential oil nanoparticles using chitosan-arabic gum complex nanocarrier with ionic gelation method. International Journal of Food Science 2022;2022.
[35] Granata G, Stracquadanio S, Leonardi M, Napoli E, Malandrino G, Cafiso V, Stefani S, Geraci C. Oregano and thyme essential oils encapsulated in chitosan nanoparticles as effective antimicrobial agents against foodborne pathogens. Molecules 2021;26.
[36] Mohammady M, Mohammadi Y, Yousefi G. Freeze-Drying of pharmaceutical and nutraceutical nanoparticles: the effects of formulation and technique parameters on nanoparticles characteristics. J Pharm Sci 2020;109:3235–47.
[37] Kristich CJ, Rice LB, Arias CA. Enterococcal infection-treatment and antibiotic resistance. In: Gilmore MS, Clewell DB, Ike Y, Shankar N, editors. Enterococci: from commensals to leading causes of drug resistant infection. Boston; 2014.
[38] Gomes BP, Pinheiro ET, Sousa EL, Jacinto RC, Zaia AA, Ferraz CC, de Souza-Filho FJ. *Enterococcus faecalis* in dental root canals detected by culture and by polymerase chain reaction analysis. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2006;102:247–53.
[39] Matsumoto-Nakano M. Role of Streptococcus mutans surface proteins for biofilm formation. Jpn Dent Sci Rev 2018;54:22–9.
[40] How KY, Song KP, Chan KG. Porphyromonas gingivalis: an overview of periodontopathic pathogen below the gum line. Front Microbiol 2016;7.
[41] Aleksijevic LH, Aleksijevic M, Skrlec I, Sram M, Sram M, Talapko J. Porphyromonas gingivalis virulence factors and clinical significance in periodontal disease and coronary artery diseases. Pathogens 2022;11.
[42] Becker RE, Bubeck Wardenburg J. Staphylococcus aureus and the skin: a longstanding and complex interaction. Skinmed 2015;13:111–9. quiz 20.
[43] Kim SY, Kim YK, Jang YS, Lee MH. Enhancement of biofunctionalization by loading Manuka oil on TiO2 nanotubes. Nanomaterials 2022;12.
[44] Christoph F, Kaulfers PM, Stahl-Biskup E. A comparative study of the in vitro antimicrobial activity of tea tree oils s.I. with special reference to the activity of β-triketones. Planta Med 2000;66:556–60.
[45] Chen C, Duncan WJ, Hughes-Medlicott NJ, Alhamdani G, Coates DE. Chitosan-Based nanoencapsulation of mānuka oil for periodontal treatment. Int J Mol Sci 2025;26.
[46] Mathew C, Tesfaye W, Rasmussen P, Peterson GM, Bartholomaeus A, Sharma M, Thomas J. Mānuka oil—A review of antimicrobial and other medicinal properties. Pharmaceuticals 2020;13:1–26.
[47] Yilmaz Atay H. Antibacterial activity of chitosan-based systems. Functional Chitosan 2020:457–89.
[48] Jesus S, Marques AP, Duarte A, Soares E, Costa JP, Colaco M, Schmutz M, Som C, Borchard G, Wick P, Borges O. Chitosan nanoparticles: shedding light on immunotoxicity and hemocompatibility. Front Bioeng Biotechnol 2020;8:100.
[49] Mokhtari H, Bahari M, Yeganeh F. Chitosan-based biomaterials in regenerative medicine: optimizing mesenchymal stem cell viability and function. Stem Cell Rev Rep 2025;21:2010–30.
[50] Weng Z, Wang Y, Ouchi T, Liu H, Qiao X, Wu C, Zhao Z, Li L, Li B. Mesenchymal Stem/Stromal cell senescence: Hallmarks, mechanisms, and combating strategies. Stem Cells Transl Med 2022;11:356–71.