J Herbmed Pharmacol. 2023; 12(2): 223-227.

http://www.herbmedpharmacol.com doi: 10.34172/jhp.2023.23

Journal of Herbmed Pharmacology

Antibacterial and cytotoxicity of chitosan nanocomposite loaded with thymol against some cariogenic bacteria

Matin Kordestani[1,2], Marzieh Rashidipour[1], Hossein Mahmoudvand[1], Shokofeh Jalali[3], Fahimeh Kooshki[4*]

[1]Razi Herbal Medicines Research Center, Lorestan University of Medical Sciences, Khorramabad, Iran
[2]Department of Restorative Dentistry, School of Dentistry, Lorestan University of Medical Sciences, Khorramabad, Iran
[3]Student Research Committee, Lorestan University of Medical Sciences, Khorramabad, Iran
[4]Department of Pediatric Dentistry, School of Dentistry, Shahid Beheshti University of Medical Sciences, Tehran, Iran

ARTICLE INFO

Article Type:
Original Article

Article History:
Received: 29 August 2022
Accepted: 25 November 2022

Keywords:
Streptococcus mutans
Actinomyces viscosus
Oral cavity
Tooth decay
Protein leakage

ABSTRACT

Introduction: Tooth decay as the most common infectious-nutritional disease in the world. The current work aims to investigate the antibacterial effect of thymol-loaded chitosan nanocomposite (TLCN) against *Streptococcus mutans* and *Actinomyces viscosus* as the main cariogenic bacteria.

Methods: Antibacterial activity of TLCN was assessed on *S. mutans* and *A. viscosus*. The effects on protein leakage in the tested bacteria, as well as its cytotoxicity, were studied by Bradford's method and cell viability assay, respectively.

Results: The size of the nanocomposite varied from 100 to 600 nm. The best minimum inhibitory concentration (MIC) related to nanocomposite + chlorhexidine was reported 2.66 for both bacteria. TLCN dose-dependently increased the protein leakage ($P<0.05$). The 50% cytotoxic concentration (CC_{50}) of nanocomposite against on normal (HGF1-PI1) and cancer (KB) cells were 149.6 and 68.4 µg/mL, respectively.

Conclusion: TLCN, especially in combination with chlorhexidine, displayed potent antibacterial effects against the main cariogenic bacterial causes. Nevertheless, other examinations are required to illuminate the precise mechanisms and its toxicity mainly in clinical settings.

Implication for health policy/practice/research/medical education:
We revealed that thymol-loaded chitosan nanocomposite (TLCN) had promising antibacterial effects against cariogenic bacterial causes. Nevertheless, supplementary examinations are obligatory to elucidate the precise mechanisms and its toxicity.

Please cite this paper as: Kordestani M, Rashidipour M, Mahmoudvand H, Jalali S, Kooshki F. Antibacterial and cytotoxicity of chitosan nanocomposite loaded with thymol against some cariogenic bacteria. J Herbmed Pharmacol. 2023;12(2):223-227. doi: 10.34172/jhp.2023.23.

Introduction

Tooth decay as the most common infectious-nutritional disease in the world (1,2) is associated with the dissolution and destruction of calcareous tissues of teeth by a mass of bacteria that are able to create an environment with sufficient acidic (acidogenic) conditions to remove the mineral content of dental tissues (3-5).

Bacteria in the mouth such as *Streptococcus mutans* and *Actinomyces viscosus*, by metabolizing carbohydrates, lead to acid production, demineralization, and tooth decay (6). The bacterium, as the main etiological factor in tooth decay, can be stable in the environment, including mucosal surfaces exposed to salivary flow, with colony formation or free life in saliva and uniform proliferation but other bacteria cannot survive freely by duplication and must attach to the mucosal surface (7).

Nanotechnology, as a part of advanced technology related to the products comprising nanoparticles, can be used in a variety of industrial and pharmaceutical applications (8,9). Since the last decade, the use of biopolymers has attracted a lot of interest in scientific and industrial research. Unlike synthetic polymers derived from petroleum derivatives, biopolymers are very good alternatives. The reason for the tendency to them can be related to the low cost of extracting them from renewable sources and on the other hand to their non-toxic and nature-friendly nature (10).

Studies have shown that among the biopolymers used in the preparation of bio-nanocomposites, chitosan has been the focus of researchers (11). Chitosan ($C_6H_{11}NO_4$),

***Corresponding author**: Fahimeh Kooshki,
Email: dr.kooshki@gmail.com

because of its low toxicity, economic viability, high efficacy, and the possibility of preparing several derivatives from it, is very important (12). With its unique structure, this biopolymer has found wide application in agriculture, food industry, cosmetics, water purification, biotechnology, textile, pharmaceutical, medicine, and biomedicine. Low price, high biocompatibility, low toxicity, as well as acceptable antimicrobial and anti-allergic properties of chitosan, have been emphasized as a special biopolymer (13). Thymol, as one of the constituents resulting from herb essential oils, has some pharmacological and therapeutic properties, such as antimicrobial, antioxidant, anticancer, and anti-inflammatory activities (14,15). Studies also have shown the positive effects of thymol on tooth decay and the bacteria that cause it. Thymol is one of the effective compounds in some disinfectant mouthwashes that fight against dental plaque (16,17). This work was intended to assess the antibacterial properties of thymol-loaded chitosan nanocomposite (TLCN) on some bacteria of caries agents, including *S. mutans* and *A. viscosus*.

Materials and Methods

Production and separation of nanocomposites

The nanocomposite was synthesized by the process defined earlier (18) with some adjustments. The characterization of TLCN was considered through a scanning electron microscope (SEM), nano-sizer-zeta-sizer, and Fourier-transform infrared spectroscopy (FTIR).

Bacterial strains

Actinomyces viscosus strain with PTCC 1202 and *S. mutans* strain with ATCC 35668 and were kept in Tryptic Soy Broth (TSB) at 37°C in 5% CO_2. Then, the McFarland 0.5 solution was provided as previously described (18).

Antibacterial activity

In this work to assess the antibacterial effects, we determined the minimum inhibitory concentration (MIC) of TLCN on the tested bacteria through broth micro-dilution method based on the Clinical and Laboratory Standards Institute (CLSI) procedures (19). The last concentration of the TLCN without any bacteria was stated as the minimum bactericidal concentration (MBC) of TLCN (20).

Effects of nanocomposite on protein leakage in bacteria

The activity of the synthesized nanocomposite on protein leakage was studied as previously elucidated (21). In summary, the tested bacteria were exposed to TLCN for 2 hours. After that, the upper phase (50 µL) was mixed with 950 µL of Bradford reagent. Bradford's method was then applied for assessing the protein content by reading its absorbance at 595 nm (BioTek, USA).

Cytotoxicity effects

Cancer (KB) and normal (HGF1-PI1) cell lines were prepared from the Pasture Institute of Iran, to evaluate the cytotoxicity effects of nanocomposite through the cell viability assay (22,23). Firstly, the cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), improved with fetal bovine serum (FBS) (15%), pen/strep (100 µg/mL). They were treated with the different concentrations of nanocomposite for 72 hours at 37°C with 5% CO_2, and 50% cytotoxic concentration (CC_{50}) was then determined.

Statistical analysis

SPSS software (version 21.0) was applied for statistical analysis. $P<0.05$ was reported as a significant level.

Results

Nanocomposite characterization

The synthesized TLCN were uniform in shape with sizes from 100-600 nm with a mediocre size of 295 nm (Figure 1). The synthesized nanoparticles showed peaks at 3142 cm, which were linked to the amino group, peaks in the ranges of 2967 and 2785 were connected with the C-H

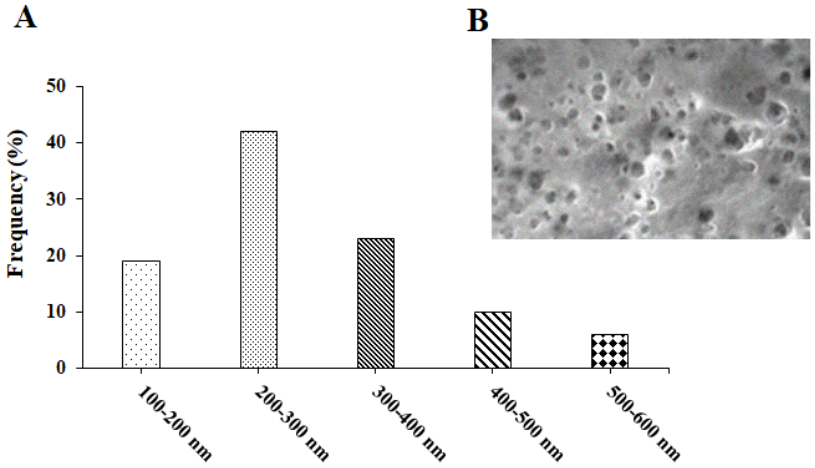

Figure 1. Size (A) and scanning electron microscope analysis (B) of thymol-loaded chitosan nanocomposite.

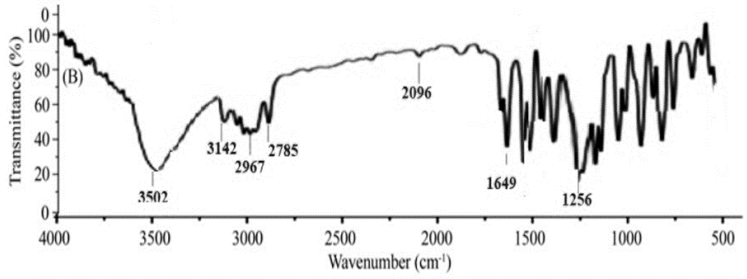

Figure 2. Fourier-transform infrared spectroscopy spectrum of the synthesized nanocomposite.

bond, peaks at 1649 was carboxyl group of chitosan, and the peaks of 580 were the ether bonds (C-O-C), respectively (Figure 2). The existence of these peaks in nanocomposite with no development of covalent bonding shows the synthesis of chitosan nanocomposite loaded with thymol. Based on the obtained findings, the presence of a peak change and crushing was also detected at 3502 cm, which was associated to O-H and N-H stretching group and could be caused by the interactions among the molecules.

Antibacterial effects on cariogenic bacteria

Table 1 shows the results of MIC and MBC after 3 replications for nanocomposite, chlorhexidine, and nanocomposite + CLX combination on both bacterial species. The best MIC related to TLCN + CLX was shown 2.66 µg/mL for both tested bacteria. The TLCN + CLX in evaluation with CLX had a better antibacterial effect ($P < 0.05$).

Effects of nanocomposite on protein leakage in bacteria

The protein leakage after treating *S. mutans* and *A. viscosus* with TLCN at 1/2 MIC, 1/3 MIC, and 1/4 MIC is shown in Figure 3. The findings exhibited that the exposure of the tested bacteria to TLCN, dose-dependently increased the protein leakage; whereas at 1/2 and 1/3 MIC markedly ($P < 0.05$) elevated the protein leakage in the tested bacteria.

Cytotoxicity effects of TLCN on cell viability

The cytotoxicity activity of TLCN was studied on HGF1-PI1 and KB cells after 72 hours incubation (Figure 4). The MTT results exhibited that the CC_{50} of nanocomposite against KB and HGF1-PI1 were 68.4, and 149.6 µg/mL, respectively.

Discussion

We revealed that the lowest MIC of TLCN + chlorhexidine was 2.66 µg/mL for the tested bacteria, indicating both bacteria displayed the equal sensitivity to this nanocomposite. Similarly, the TLCN + chlorhexidine nanocomposite showed the lowest MBC at 2.66 µg/mL for both bacteria. Therefore, it can be concluded that TLCN at 2.66 µg/mL, in addition to inhibiting the growth of bacteria, also caused their death. So far, several studies confirmed the role of thymol in controlling bacteria responsible for tooth decay (24). Khan et al showed that thymol inhibits growth and reduces biofilm formation by *S. mutans* with the IC_{50} 54 µg/mL (25). Laboratory results obtained from the study of Alvarez Echazú et al showed that thymol-chitosan hydrogels have antimicrobial effects against *Staphylococcus aureus* and *S. mutans* for 72 hours and antioxidant effects for 24 hours (26). In a study by Wattanasatcha et al (27), thymol in sub-micron sizes had antibacterial effects and inhibited the growth of *E. coli, S. aureus* and *P. aeruginosa*. In the study by Lee et al (28), a

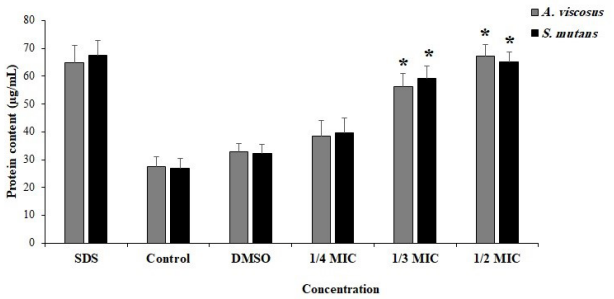

Figure 3. Effects of the thymol-loaded chitosan nanocomposite on the leakage of protein in the tested bacteria at 1/4 of the minimum inhibitory concentration (MIC), 1/3 MIC, and ½ 2 MIC. (mean ± SD). *$P < 0.05$ compared to the control group. DMSO: dimethyl sulfoxide; SDS: sodium dodecyl sulfate.

Table 1. Antibacterial effect of the synthesized nanocomposite on *Streptococcus mutans* and *Actinomyces viscosus*

Drug	Minimum bactericidal concentration (µg/mL)		Minimum inhibitory concentration (µg/mL)	
	Actinomyces viscosus	*Streptococcus mutans*	*Actinomyces viscosus*	*Streptococcus mutans*
Nanocomposite	8.0±0.0	7.3±1.15	7.3±1.15	6.6±1.154
Chlorhexidine	5.33±2.4	4.66±1.15	5.33±2.4	4.66±1.15
Nanocomposite + Chlorhexidine	2.66±1.154*	2.66±1.154*	2.66±1.154*	2.66±1.154*

*$P < 0.05$ significant difference compared with the chlorhexidine.

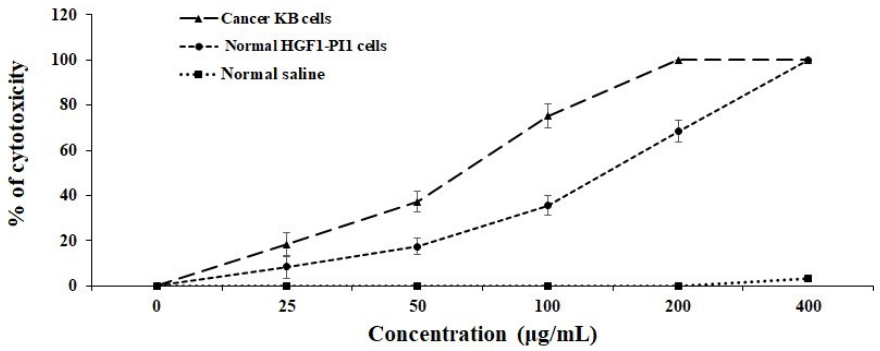

Figure 4. The cytotoxicity activity of thymol loaded chitosan nanocomposite on human normal and oral cancer cell lines. Results are mean ± SD (n = 3).

combination of clove oil and thymol displayed a potent antimicrobial activity against S. *mutans* and *S. sobrinus*. Zhu et al (29) also showed the promising antibacterial effects of thymol microencapsulation in poly (lactide-co-glycolide) (PLGA) against *E. coli* and *S. aureus*.

Considering the antimicrobial mechanisms of these nanocomposites, we found that exposure of tested bacteria with nanocomposite dose-dependently increased the protein leakage; whereas at 1/2 and 1/3 MIC markedly (P<0.05) elevated the protein leakage in the tested bacteria compared with the control group. Studies have shown that the antimicrobial activity of thymol is through the cytoplasmic membrane disruption and outflow of intracellular substances, which result in bacterial death (30). One of the main mechanism actions of thymol, which leads to the reduction and inhibition of bacterial growth, is related to its effects on the bacterial cell membrane, because thymol is a strong compound that is harmful to the outer membrane (30,31).

As the first step to evaluate the toxicity of new agents that are used as mouthwashes for the treatment and prevention of tooth decay, we tested the cytotoxicity effects of the synthesized thymol loaded chitosan nanocomposite against human normal (HGF1-PI1) and cancerous cells (KB) of the mouth. Concerning the cytotoxic activity of thymol loaded chitosan nanocomposite, we showed that the CC_{50} value of nanocomposite against KB and HGF1-PI1 cells were 149.6 and 68.4 µg/mL, respectively, representing the cytotoxic effects of TLCN on cancerous cells; however, TLCN was nontoxic for normal cells.

Conclusion

These results revealed that TLCN, especially in combination with chlorhexidine, displayed potent antibacterial effects against *S. mutans* and *A. viscosus* as the main cariogenic bacterial causes. However, extra examinations are mandatory to clarify the exact mechanisms and its toxicity mainly in clinical settings.

Authors' contribution

KM supervised the study. MR, PS, SJ, and FK reviewed and contributed to data collection and preparation of the manuscript. The first draft was prepared by FK and MK. All authors read the final version and confirmed it for publication.

Conflict of interests

The authors declare no conflict of interest.

Ethical considerations

This project was approved by the ethics committee of Lorestan University of Medical Sciences with the ethics ID IR.LUMS.REC.1400.272.

Funding/Support

None.

References

1. Selwitz RH, Ismail AI, Pitts NB. Dental caries. Lancet. 2007;369(9555):51-9. doi: 10.1016/s0140-6736(07)60031-2.
2. Petersen PE, Bourgeois D, Ogawa H, Estupinan-Day S, Ndiaye C. The global burden of oral diseases and risks to oral health. Bull World Health Organ. 2005;83(9):661-9.
3. Yadav K, Prakash S. Dental caries: review. Asian J Biomed Pharm Sci. 2016;6(53):1-7.
4. Beighton D. Can the ecology of the dental biofilm be beneficially altered? Adv Dent Res. 2009;21(1):69-73. doi: 10.1177/0895937409335641.
5. Mitchell TJ. The pathogenesis of streptococcal infections: from tooth decay to meningitis. Nat Rev Microbiol. 2003;1(3):219-30. doi: 10.1038/nrmicro771.
6. Marsh PD. Microbiologic aspects of dental plaque and dental caries. Dent Clin North Am. 1999;43(4):599-614.
7. McNeil SE. Nanotechnology for the biologist. J Leukoc Biol. 2005;78(3):585-94. doi: 10.1189/jlb.0205074.
8. Emerich DF, Thanos CG. Nanotechnology and medicine. Expert Opin Biol Ther. 2003;3(4):655-63. doi: 10.1517/14712598.3.4.655.
9. Kim BY, Rutka JT, Chan WC. Nanomedicine. N Engl J Med. 2010;363(25):2434-43. doi: 10.1056/NEJMra0912273.
10. Shamshina JL, Berton P, Rogers RD. Advances in functional chitin materials: a review. ACS Sustain Chem Eng. 2019;7(7):6444-57. doi: 10.1021/acssuschemeng.8b06372.
11. Goy RC, de Britto D, Assis OB. A review of the antimicrobial

activity of chitosan. Polímeros. 2009;19(3):241-7. doi: 10.1590/s0104-14282009000300013.
12. Kou SG, Peters LM, Mucalo MR. Chitosan: a review of sources and preparation methods. Int J Biol Macromol. 2021;169:85-94. doi: 10.1016/j.ijbiomac.2020.12.005.
13. Escobar A, Pérez M, Romanelli G, Blustein G. Thymol bioactivity: a review focusing on practical applications. Arab J Chem. 2020;13(12):9243-69. doi: 10.1016/j.arabjc.2020.11.009.
14. Parsaei P, Bahmani M, Naghdi N, Asadi-Samani M, Rafieian-Kopaei M. A review of therapeutic and pharmacological effects of thymol. Der Pharm Lett. 2016;8(2):150-4.
15. Marchese A, Orhan IE, Daglia M, Barbieri R, Di Lorenzo A, Nabavi SF, et al. Antibacterial and antifungal activities of thymol: a brief review of the literature. Food Chem. 2016;210:402-14. doi: 10.1016/j.foodchem.2016.04.111.
16. Karimi N, Jabbari V, Nazemi A, Ganbarov K, Karimi N, Tanomand A, et al. Thymol, cardamom and *Lactobacillus plantarum* nanoparticles as a functional candy with high protection against Streptococcus mutans and tooth decay. Microb Pathog. 2020;148:104481. doi: 10.1016/j.micpath.2020.104481.
17. Hu Y, Du Y, Wang X, Feng T. Self-aggregation of water-soluble chitosan and solubilization of thymol as an antimicrobial agent. J Biomed Mater Res A. 2009;90(3):874-81. doi: 10.1002/jbm.a.31871.
18. Azadbakht K, Hadipour S, Rashidipour M, Sepahvand A. Antifungal effects of thymol-loaded chitosan nanocomposite alone and in combined with nystatin against *Candida albicans*, a major cause of oral candidiasis. Glob J Med Pharm Biomed Update. 2022;17(7):1-6. doi: 10.25259/gjmpbu_18_2022.
19. Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement. Wayne, PA: CLSI; 2012.
20. Ebrahimi K, Shiravand S, Mahmoudvand H. Biosynthesis of copper nanoparticles using aqueous extract of *Capparis spinosa* fruit and investigation of its antibacterial activity. Marmara Pharm J. 2017;21(4):866-71.
21. Ghorbanizadeh S, Karami F, Delfani S, Shakibaie M, Razlansari A, Rezaei F. Antibacterial effects and cellular mechanisms of iron oxide magnetic nanoparticles coated by piroctone olamine against some cariogenic bacteria. Ann Med Surg (Lond). 2022;81:104291. doi: 10.1016/j.amsu.2022.104291.
22. Albalawi AE, Khalaf AK, Alyousif MS, Alanazi AD, Baharvand P, Shakibaie M, et al. Fe3O4(@)piroctone olamine magnetic nanoparticles: synthesize and therapeutic potential in cutaneous leishmaniasis. Biomed Pharmacother. 2021;139:111566. doi: 10.1016/j.biopha.2021.111566.
23. Mahmoudvand H, Tavakoli R, Sharififar F, Minaie K, Ezatpour B, Jahanbakhsh S, et al. Leishmanicidal and cytotoxic activities of *Nigella sativa* and its active principle, thymoquinone. Pharm Biol. 2015;53(7):1052-7. doi: 10.3109/13880209.2014.957784.
24. Liao G, Tang X. Mining the microbial chemistry behind tooth decay. Biochemistry. 2022;61(24):2779-81. doi: 10.1021/acs.biochem.1c00652.
25. Khan ST, Khan M, Ahmad J, Wahab R, Abd-Elkader OH, Musarrat J, et al. Thymol and carvacrol induce autolysis, stress, growth inhibition and reduce the biofilm formation by *Streptococcus mutans*. AMB Express. 2017;7(1):49. doi: 10.1186/s13568-017-0344-y.
26. Alvarez Echazú MI, Olivetti CE, Anesini C, Perez CJ, Alvarez GS, Desimone MF. Development and evaluation of thymol-chitosan hydrogels with antimicrobial-antioxidant activity for oral local delivery. Mater Sci Eng C Mater Biol Appl. 2017;81:588-96. doi: 10.1016/j.msec.2017.08.059.
27. Wattanasatcha A, Rengpipat S, Wanichwecharungruang S. Thymol nanospheres as an effective anti-bacterial agent. Int J Pharm. 2012;434(1-2):360-5. doi: 10.1016/j.ijpharm.2012.06.017.
28. Lee JS, Choi YS, Lee HG. Synergistic antimicrobial properties of nanoencapsulated clove oil and thymol against oral bacteria. Food Sci Biotechnol. 2020;29(11):1597-604. doi: 10.1007/s10068-020-00803-w.
29. Zhu Z, Min T, Zhang X, Wen Y. Microencapsulation of thymol in poly(lactide-co-glycolide) (PLGA): physical and antibacterial properties. Materials (Basel). 2019;12(7):1133. doi: 10.3390/ma12071133.
30. Li J, Chang JW, Saenger M, Deering A. Thymol nanoemulsions formed via spontaneous emulsification: Physical and antimicrobial properties. Food Chem. 2017;232:191-7. doi: 10.1016/j.foodchem.2017.03.147.
31. Xu J, Zhou F, Ji BP, Pei RS, Xu N. The antibacterial mechanism of carvacrol and thymol against *Escherichia coli*. Lett Appl Microbiol. 2008;47(3):174-9. doi: 10.1111/j.1472-765X.2008.02407.x.