Enhancement the effect of some phenolic compounds against bacterial pathogens usingnanotechnology

Thesis Presented by

Assistant Researcher/

Asmaa Mohammed Heneter Helwy

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Under Supervision of

Prof. Dr. Talaat Sayed Aly El-Khateib

Professor of Meat Hygiene
Department of Food Hygiene
Faculty of Veterinary Medicine
Assiut University.

Prof. Dr.Ashraf Mohamed Abd El-Malek

Professor of Meat Hygiene
Department of Food Hygiene
Faculty of Veterinary Medicine
Assiut University.

Dr.Mahmoud Ammar Mohamed Ammar

Senior Researcher of Meat Hygiene
Animal Health Research Institute
Department of Food Hygiene
Assiut Lab

Dr.Ahmad Mohamed Ahmad Abo Markeb

Lecturer of Chemistry
Department of Chemistry
Faculty of Science
Assiut University

Department of Food Hygiene
Faculty of Veterinary Medicine
Assiut University
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List of abbreviations

Abbreviation	Meaning
ADI	Acceptable Daily Intake
AOF	Animal Origin Food
APHA	American Public Health Association
CDC	Centers for Diseases Control and Prevention
Cfu	Colony forming unit
CIREPBN	Cosmetic Ingredient Review Expert Panel Bindu Nair
CsNPs	Chitosan nanoparticles
CPC	Chitosan-Phytochemical Conjugates
CS	Chitosan
DMSO	Dimethylsulfoxide
EE	Encapsulation Efficiency
EFSA	European Food Safety Authority
EO	Essential Oil
ERS	Economic Research Service
ES	Egyptian Standards Specifications
EUCAST	European Committee for Antimicrobial SusceptibilityTesting
FAO	Food and Agriculture Organization
FBCI	Fractional Bactericidal Concentration Index
FDA	Food and Drug Administration
FEMA	Flavoring Extract Manufacturers Association
FERG	Foodborne Disease Burden Epidemiology Reference Group
FICI	Fractional Inhibitory Concentration Index
FSAI	Food Safety Authority of Ireland
FTIR	Fourier transform infrared
GRAS	Generally Recognized As Safe
Abbreviation	**Meaning**
HRTEM	High Resolution Transmission Electron Microscopy
ICMSF	International Commission on Microbiological Specifications for
IFSAC	Interagency Food Safety Analytics Collaboration

IPCS	International Programme on Chemical Safety
ISO	International Organization for Standardization
LMWC	Low Molecular Weight Chitosan
LPSPs	Lightly Preserved Seafood Products
MARD	Ministry of Agriculture and Rural Development
MIC	Minimum Inhibitory Concentration
MLC	Minimum lethal Concentration
NPs	Nanoparticles
NSAI	National Standards Authority of Ireland
RASFF	Rapid Alert System for Food and Feed
TEM	Transmission Electron Microscope
TBHQ	Tertiary Butylhydroquinone
USDA	United States Department of Agriculture
USDA-FSIS	United States Department of Agriculture-Food Safety and Inspection Services
WHO	World Health Organization

Summary

Food safety is one of the major challenges for the 21s century implying a significant redirection of food microbiologist efforts in many parts of the world toward the prevention of food borne diseases.Great efforts are directed to implement antimicrobial strategies to overcome the contamination of fish with *Salmonella* spp. and *L. monocytogenes*through its procession from farm to fork.

The increased resistance of bacteria to traditional antimicrobial resulted argent demand in the area of food processing to recent andnatural antimicrobials to control these potential pathogens.Phenolic compounds are bioactive substances occurring widely in food plants. The phenolic fraction of plant extracts (secondary metabolites and active component) has been linked to their antimicrobial activity as natural and safer alternatives to chemical disinfectants in food systems. Also, it is of low cost.

In the present study,the antimicrobial properties of some phenolic compounds (thymol, tannic acid, gallic acid, curcumine, coumarin, cinnamic acid, benzoic acid and ascorbic acid), their combinations, chitosan, its nanoparticles and thymol loaded chitosan nanoparticles were studied in microbiological media and in fish matrixagainst food pathogens (*S.* Typhimurium and *L.monocytogenes*).

The invitro antibacterial activity was evaluated using visual method and four spectrophotometric methods.By visual assay, the MICs against *S.* Typhimuriumvaried from 0.078 to 10 mg/ml while the corresponding values for spectrophotometric methods were 0.156 to 10 mg/ml. The most powerful effect was obtained by thymol. It could inhibit Salmonella at concentration of (0.078 mg/ml by visual method and 0.156 mg/ml by spectrophotometric methods with growth inhibition 100%) and kill it at concentration of 0.078

mg/ml. Also, benzoic acid appeared powerful killing ability against Salmonella where it was inhibitor at level of 1.25 mg/ml and cidal at 2.5 mg/ml. Some other phenolics showed both inhibitory and cidal effects but at higher concentrations than thymol and benzoic acid.

Regarding *L. monocytogenes*; it was affected by most of the tested phenolics in varying degrees. The most potent effect obtained by thymol followed by benzoic acid and cinnamic acid where the MICs values were (0.156 mg/mlby all methods for thymol with growth inhibition 100%), (1.25-2.5mg/ml for benzoic acid and cinnamic acid) and MLCs were (0.156 mg/ml for thymol), (2.5 mg/ml for benzoic acid) and (5 mg/ml for cinnamic acid).

The results indicated that cinnamic acid had a significant inhibitory effect on both Gram positive and Gram negative bacteria. Its MICs against *S.* Typhimurium were 5 mg/ml by visual method, method 1, method 2 and method 4 and 2.5 mg/ml by method 3 with growth inhibition 90.4% while the MLC was 5 mg/ml. Against *L. monocytogenes*, its MICs (1.25 mg/ml by visual method, method 1 and method 3) inhibit growth by 98.2% and 2.5 mg/ml by method 2 and method 4 inhibit growth by 100%).

The results indicated that tannic acid had an inhibitory effect on *S.* Typhimurium with MIC of 10 mg/ml by visual method and method 2 of spectrophotometric methods with a growth inhibition of 100%. The highest concentration tested in this study (10 mg/ml) wasn't lethal to *S.* Typhimurium. Comparatively, it was inert against *L. monocytogenes.*

The results of in vitro antimicrobial activity of gallic acid against *S.* Typhimurium were 10 mg/ml as MIC by visual method, method 1, method 2 and method 4 and 5 mg/ml by method 3 with growth inhibition 94.1% while the MLC was 10 mg/ml.

Regarding *L. monocytogenes*, gallic acid presented an antibacterial activity against it with MIC of 5 mg/ml by visual method, method 2 and method 3 with growth inhibition 100% and 10 mg/ml by method 1 and method 4. Also, the MLC was 10 mg/ml.

When testing curcumine against *S.* Typhimurium, its MIC was 10 mg/ml by visual method, while by method 2 and method 3, its MIC was 5 mg/ml with a growth inhibition 100%. MICs by method 1 and method 4 in addition to MLC were not detected. Also, MICs and MLC of curcumine against *L. monocytogenes*were not detected in this study.

At a concentration of 2.5 mg/ml as a MIC, coumarin inhibited the growth of *S.* Typhimurium by 100% where the MLC was 10 mg/ml. Comparatively, the growth of *L. monocytogenes*was inhibited at a concentration of 2.5 mg/ml as MIC by 100 % and the MLC was 5 mg/ml.

Benzoic acid exhibited a marked antibacterial activity against *S.* Typhimurium and *L. monocytogenes* where its MICs against *S.* Typhimurium were 1.25 mg/ml by visual method and method 1 with growth inhibition 98.2% and 2.5 mg/ml by method 2 and method 4 with growth inhibition 100% and 0.625 mg/ml by method 3 with growth inhibition 85.9% while MLC was 2.5 mg/ml.

MICs of benzoic acid against *L. monocytogenes* were 1.25 mg/ml by visual method, method 1 and method 3 with growth inhibition 98.2% and 2.5 mg/ml by method 2 and method 4 with growth inhibition 100% also; the MLC was 2.5 mg/ml.

The MICs of ascorbic acid against *S.* Typhimurium were 5 mg/ml by visual method and 2.5 mg/ml by spectrophotometric methods with growth inhibition 100% while the MLC was 10 mg/ml. It presented an antibacterial

activity against *L. monocytogenes*with MIC of 5 mg/ml by all used methods and MLC of 10 mg/ml.

When mixing thymol and benzoic acid at their MICs against *S.* Typhimurium, the FICI revealed an indifferent effect. The MIC obtained by effect of the combination was 0.078 mg/ml thymol: 1.25 mg/ml benzoic acid by visual method while it couldn't be detected by spectrophotometric methods. The MLC was more than (0.078 mg/ml thymol: 1.25 mg/ml benzoic acid).

With mixing thymol and benzoic acid at equal proportions against *S.* Typhimurium the FICI and FBCI were indifferent). The MIC obtained by the combination was 0.078 mg/ml each by visual method; also the MLC was 0.078 mg/ml each. The MIC was 0.156 mg/ml each by method 1 and method 3 with growth inhibition 99.7% and 0.312 mg/ml each by method 2 and method 4with growth inhibition 100%.

As for *S.* Typhimurium, when mixing thymol and benzoic acid at their MICs against *L. monocytogenes*, the FICI indicated additive effect while FBCI indicated indifferent effect. The obtained MIC of the combination was 0.078 mg/ml thymol: 0.625 mg/ml benzoic acid by visual method, method 1 and method 3 with growth inhibition 98.5% and 0.156 mg/ml thymol: 1.25 mg/ml benzoic acid by method 2 and method 4 with growth inhibition 100%. The MLC was 0.156 mg/ml thymol:1.25 mg/ml benzoic acid.

With mixing thymol and benzoic acid at equal proportions,at MIC against *L. monocytogenes*, the FICI indicated indifferent effect. The MIC obtained by the combination was 0.156 mg/ml each by all methods of evaluation with growth inhibition 100% while MLC was 0.312 mg/ml each. The FBCI indicated antagonism effect of the combination.

With mixtures of thymoland cinnamic acid at their MICs against *L. monocytogenes*, the FICI indicated additive effect. The MIC of the

combination was 0.156 mg/ml thymol: 1.25 mg/ml cinnamic acid by visual method, method 2 and method 4 with a growth inhibition 100% and 0.078 mg/ml thymol: 0.625 mg/ml cinnamic acid by method 1 and method 3 with a growth inhibition 86.4%. The applied combinations didn't have cidal effect.

With mixing thymol and cinnamic acid at equal proportions, antagonism was observed at FIC and FBC indexes against *L. monocytogenes*. The MIC of the combination was 0.312 mg/ml each by visual method; also MLC was 0.312 mg/ml each while MIC couldn't be detected by spectrophotometric methods.

With mixtures of cinnamic and benzoic acids at their MICs and at equal proportions, FICI indicated additive effect against *L. monocytogenes*. The MIC of the combination was 0.625 mg/ml (each) by visual and spectrophotometric methods with a growth inhibition 100%. The combination had a cidal effect where the MLC was 0.625 mg/ml each. Also, the two phenolics enhanced each other and resulted a synergistic effect.

Some phenolic compounds like vitamin-C are good antioxidants.Vitamin-C is called ascorbic acid. It is water soluble. It is able to react with aqueous free radicals and reactive oxygen to neutralize them.

By addition of ascorbic acid to thymol-benzoic acid combinations (each at MIC) against *S*. Typhimurium, the FICI was indifferent. The MIC resulted by the combination was (0.078 mg/ml thymol: 1.25 mg/ml benzoic acid: 2.5 mg/ml ascorbic acid) by visual method, method 1, method 2 and method 4 with growth inhibition 100%. The corresponding MIC value was (0.039 mg/ml thymol: 0.625 mg/ml benzoic acid: 1.25 mg/ml ascorbic acid) by method 3 with growth inhibition 85.11%. The obtained MLC was (0.078 mg/ml thymol: 1.25 mg/ml benzoic acid: 2.5 mg/ml ascorbic acid).

When thymol, benzoic acid and ascorbic acid were mixed at equal proportions against *S*. Typhimurium, the FICI was indifferent. The MIC

outcome of the combination was (0.156 mg/ml each) by visual method; also the MLC was (0.156 mg/ml each). The MIC by spectrophotometric methods was (0.312 mg/ml each) with growth inhibition 100%.

With addition of ascorbic acid to thymol-benzoic acid combinations (eachat MIC), the compounds enhanced each other and synergy was observed against *L. monocytogenes*. The MIC of the combination was (0.039 mg/ml thymol: 0.312 mg/ml benzoic acid: 1.25 mg/ml ascorbic acid) by visual method with a growth inhibition 99.1%. While it was (0.019 mg/ml thymol: 0.156 mg/ml benzoic acid: 0.625 mg/ml ascorbic acid) by method 1 and method 3. By method 2 and method 4, the recorded MIC was (0.078 mg/ml thymol: 0.625 mg/ml benzoic acid: 2.5 mg/ml ascorbic acid) with growth inhibition 100% also the MLC was (0.078 mg/ml thymol: 0.625 mg/ml benzoic acid: 2.5 mg/ml ascorbic acid).

When thymol, benzoic acid and ascorbic acid were mixed at equal proportions against *L. monocytogenes*, the FICI was indifferent. The resulted MIC of the combination was (0.078 mg/ml each) by visual method while MIC by method 1 and method 3 was (0.156 mg/ml each) with a growth inhibition 97.6%. By method 2 and method 4, it was (0.312 mg/ml each) with growth inhibition 100%. The MLC was 0.156 mg/ml each.

With addition of ascorbic acid to thymol-cinnamic acid combinations (each at MIC), the enhancement of action resulted synergism against *L. monocytogenes*. The MIC gained by the combination was (0.078 mg/ml thymol: 0.625 mg/ml cinnamic acid: 2.5 mg/ml ascorbic acid) by visual method, method 2 and method 4 with growth inhibition 100%. While it was (0.039 mg/ml thymol: 0.312 mg/ml cinnamic acid: 1.25 mg/ml ascorbic acid) by method 1 and method 3 with growth inhibition 95.6%. The MLC was (0.156 mg/ml thymol: 1.25 mg/ml cinnamic acid: 5 mg/ml ascorbic acid).

When thymol, cinnamic acid and ascorbic acid were mixed at equal proportions, antagonism was observed against *L. monocytogenes*. The MIC of the combination was 0.625 mg/ml each by visual method but it couldn't be detected by spectrophotometric methods. The MLC was 1.25 mg/ml each.

With addition of ascorbic acid to cinnamic-benzoic acids combinations (each at MIC), potentiation occurred and the sum of effect resulted synergy against *L. monocytogenes*. The MIC achieved by the combination was (0.156 mg/ml cinnamic acid: 0.156 mg/ml benzoic acid: 0.625 mg/ml ascorbic acid) by visual method, method 1 and method 3 with growth inhibition 92.6%. While it was (0.625 mg/ml cinnamic acid: 0.625 mg/ml benzoic acid: 2.5 mg/ml ascorbic acid) by method 2 and method 4 with growth inhibition 100%. Also, MLC was (0.625 mg/ml cinnamic acid: 0.625 mg/ml benzoic acid: 2.5 mg/ml ascorbic acid).

When cinnamic, benzoic and ascorbic acids were mixed at equal proportions against *L. monocytogenes*,the interpretation of FICI was indifferent. The MIC recorded for the combination was (0.625 mg/ml each) by visual method, method 1 and method 3 with growth inhibition 98.4%. The MIC by method 2 and method 4 was (1.25 mg/ml each) with growth inhibition 100%, where the MLC was also (1.25 mg/ml each).

The in vitro investigation of chitosan activity against *S.* Typhimurium revealed 0.625 mg/ml as MIC by visual and spectrophotometric methods with growth inhibition 100 %; also the MLC was 0.625 mg/ml.

Regarding*L. monocytogenes*, chitosan presented an antibacterial activity against it with MIC of 0.039 mg/ml by visual and spectrophotometric methods with growth inhibition 100 %, while the MLC was 0.078 mg/ml.

Among the phenolics studied, thymol presented the highest antibacterial activity against *S.* Typhimurium and*L. monocytogenes*, hence, thymol was

chosen forincorporation into nanocarrier polymer (chitosan)for studying antimicrobial properties both in vitro and in fish matrix.The formation of nanoparticles was proved by HRTEM technique, FTIR and XRD.

HRTEM revealed a successful preparation of nanoparticles. Obviously, thymol is present in the core of the particles and surrounded with a shell of chitosan forming self-assembly nanostructure. Moreover, the particles obtained are well dispersed with no significant of agglomeration. The size of the particle loaded with thymolwas in the range of41.97 - 64.36 nm. Whilethe size of chitosan nanoparticles as a control is in the range of61.45 - 86.95nm.

It was found that 99.54 %of the total thymol was encapsulated while loading capacity was 64.17 %.

Yield particlespercentage of thymol loaded chitosan nanoparticles was 96.3 % whileyield particles percentage of chitosan nanoparticles (control) was 99.45 %.

The zeta potential of the thymol loaded chitosan nanoparticles and chitosan nanoparticles were found to be 54.80 mV, and 34.50 mV, respectively.

It was found that a slight release of thymolfrom chitosan nanoparticles at pH 7.4 until 8 h to be 3.5 %. Then, a slight increase in the release was shown being 15.81 % after 12 h. In addition, the release was achieved 18.64 % from 12 h to 48 h which indicating the stability of thymol in chitosan nanoparticles.

The MIC of chitosan nanoparticles against *S.* Typhimuriumwas 0.8 mg/ml by visual and spectrophotometric methods with growth inhibition 100 % while the MLC was 1.6 mg/ml. It presented an antibacterial activity against *L. monocytogenes*with MIC of 0.2 mg/ml by all used methods and growth inhibition of 100 %. Also the MLC was 0.2 mg/ml.

Thymol loaded chitosan nanoparticles exhibited a marked antibacterial activity against *S.* Typhimurium and *L. monocytogenes* where itsMIC against *S.* Typhimurium was 0.8 mg/ml by visual and spectrophotometric methods with growth inhibition 100 %while MLC was 1.6 mg/ml.

The recorded MIC value of thymol loaded chitosan nanoparticles against *L. monocytogenes* was 0.05 mg/ml by visual and spectrophotometric methods with growth inhibition 100 % while the MLC was 0.4 mg/ml.

Thymol was investigated as edible coating in fish fillets. The data revealed that just after dipping in coating solution containing the *S.* Typhimurium MIC dose of thymol (0.078 mg/ml), the bacterium count significantly reduced ($p < 0.05$) and showed 83 % reduction compared to control. By 2MIC dose the reduction increased to 98 %.During refrigerator storage, *S.* Typhimuriumwas significantly reduced by MIC and 2MIC where 2MICdose was nearly steady effective.

Against, *L. monocytogenes* also thymol activity appeared at once after coating (zero h).By MIC concentration (0.156 mg/ml) the reduction in the cells was significant reached 70 % and increased to 90 % by double MIC. At the end of 24h storage, the reduction declined by 10% for both concentrations but still significant.At end of 96h of storage the achieved reductions were 67 and 97% for MIC and 2MIC doses, respectively.

By the four used concentrations, thymol coating resulted slightly alkalizing effect to fish fillets compared to control. That effect was non significant and the net pH value still within normal values. Also sensory panelists didn’t revealed significant differences in eating quality between thymol treated and untreated fillets.

Chitosan nanoparticles (CsNP) showed potential reduction against *S.* Typhimurium.A dose of MIC (0.8 mg/ml) produced potential delayed effect

while the MIC dose showed potential on both immediate and delayed effect. Salmonella cells were reduced by 20 % immediately afterCsNP MIC coating compared to a significant reduction (95%) by 2MIC dose. Against Salmonella the CsNP activity continued increasing and the effect was maximized (100% reduction) at 48h storage for MIC and during 24-48h for 2MIC dose. Then effect for both doses still nearly constant and significant till the end of storage time (96h).

Listeria cells were less sensitive to CsNP but significantly reduced in most of periods. The immediate effect of coating resulted 62 and 65% reduction compared to control for MIC (0.05 mg/ml) and 2MIC doses, respectively. By refrigeration, Listeriacells showed fluctuated response to CsNP. The CsNP coating appeared slight alkalizing effect at some sampling periods but not significantly.Also, by the four used concentrations of CsNP, the eating quality of fresh fillets was preserved during 4 days as judged by sensory panelists.

Loading thymol on CsNP increased the safety of fish fillets in concern with salmonella. The effect of coating of fillets with thymol loaded CsNP produced immediate reduction in salmonella cells by 67 and 83% compared to control by MIC (0.8 mg/ml) and 2MIC dose, respectively. By proceeding of time at refrigerator storage, the activity of thymol loaded CsNP against salmonella maximized by end of 24h and still potential within the three successive days.

Listeria appeared less sensitive even to thymol loaded CsNP compared to salmonella. The coating effect of thymol loaded CsNP begins immediately producing significant reduction of 62 and 63 % for MIC (0.2 mg/ml) and 2MIC doses, respectively. During term of refrigerated storage the delayed effect of thymol loaded CsNP against listeria cells decreased and reductions was only near half of MIC and 2MIC immediate reduction values.

The pH and sensory quality of fillets coated with thymol loaded CsNP recorded within range of those of fresh fish till end of 4 days of refrigerated storage.

Therefore, these findings are promising evidence to possibly aid in the prevention of microbial dissemination in foods and can be used in the industrial scale as a novel method to improve fish safetyand quality during refrigerated storage.