Journal of the Korea Academia-Industrial cooperation Society
Vol. 23, No. 2 pp. 713-722, 2022

https://doi.org/10.5762/KAIS.2022.23.2.713
ISSN 1975-4701 / eISSN 2288-4688

Synergistic Antibacterial and Antioxidant Effects of Different Molecular Weight Chitosan with Cosmetic Ingredients

In Geun Jung, Guen Moo Lee, You Jin Hwang*
Department of Biomedical Engineering, College of Health Science, Gachon University

화장품 성분과 분자량에 따른 키토산의 항균 및 항산화 시너지 효과

정인근, 이근무, 황유진*
가천대학교 의용생체공학과

Abstract This study investigates the properties of chitosan in cosmetic formulations, by examining the antibacterial and antioxidant effects when used alongside other cosmetic ingredients. Three types of chitosan with different molecular weights were examined, and five representative cosmetic ingredients used as raw materials in cosmetic formulations (1,2-hexanediol, 1,3-butylene glycol, EDTA-2Na, ethanol, and glycerol) were selected. Disc diffusion and broth microdilution assays were conducted to determine the antibacterial activity of chitosan and the cosmetic ingredients. Synergistic antibacterial effects were evaluated by applying the checkerboard synergy assay. Bacterial strains used for the experiments were Gram-positive bacteria (*Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus*) and a Gram-negative bacterium (*Escherichia coli*). Antioxidant activity was measured by the DPPH radical scavenging assay. LMWC showed potent antibacterial and antioxidant activities. Synergistic antibacterial effects (in order of potency) were determined for LMWC, HMWC, and MMWC. In particular, EDTA-2Na and ethanol showed remarkable synergistic antibacterial effects with chitosan; none of the compounds showed antagonistic effects. Our data indicate that chitosan has strong potential for use as a natural material in cosmetic formulations, and may be useful in a wide range of applications in the cosmetic industry.

요 약 본 연구에서는 키토산과 화장품 성분의 항균 및 항산화 시너지 효과를 확인하여 화장품 제형에서 키토산의 활성을 확인하고자 하였다. 키토산은 분자량이 다른 3종류를 사용하였으며, 화장품 성분으로는 제형에 대표적으로 사용되는 물질 5종(1,2-hexanediol, 1,3-butylene glycol, EDTA-2Na, ethanol, glycerol)을 선정하였다. 디스크 확산법과 액체배지 미량희석법을 수행하여 키토산 및 화장품 성분의 항균 활성을 확인하였으며, 항균 시너지 효과는 체커보드법으로 측정하였다. 본 실험에서는 실험 균주로 그람양성균인 황색포도상구균(*Staphylococcus aureus*)과 메티실린 내성 황색포도상구균(methicillin-resistant *Staphylococcus aures*), 그람음성균인 대장균(*Escherichia coli*)을 사용하였다. 항산화 활성은 DPPH 라디칼 소거능법으로 측정하였다. 저분자 키토산은 강력한 항균 및 항산화 활성을 나타냈다. 항균 시너지 효과는 저분자 키토산, 고분자 키토산, 중분자 키토산 순으로 더 강하게 나타났다. 특히, EDTA-2Na와 에탄올은 키토산과 뛰어난 항균 시너지 효과를 보였으며 키토산과 화장품 성분 간의 길항 효과는 나타나지 않았다. 키토산은 화장품 제형에 사용하기 위한 천연 물질로서 높은 잠재성을 가지며 화장품 산업에서 유용하게 사용될 수 있을 것으로 기대된다.

Keywords : Chitosan, Molecular Weight, Cosmetic Ingredients, Antibacterial, Antioxidant, Synergistic Effect

This study was supported by a grant from the RIS grant (No. 202103060001) at the Gachon University.
*Corresponding Author : You-Jin Hwang(Gachon University)
email: gene@gachon.ac.kr
Received October 14, 2021 Revised November 16, 2021
Accepted February 4, 2022 Published February 28, 2022

1. Introduction

Cosmetics are suitable substrates for microbial growth due to water, nutrients, pH, and other factors in their formulation. Microorganisms produce endotoxins and metabolites in cosmetics that can cause abrasion, irritation, and allergies on skin. Therefore, contamination by microorganisms exposes the consumer to unnecessary risk[1,2] and as a result cosmetics contain preservatives to prevent microbial growth[2]. However, chemical preservatives widely used in cosmetics, such as parabens, chlorphenesin, and phenoxyethanol, have been reported to be cytotoxic and cause skin irritation in several studies[3,4]. Consequently, the cosmetic industry has been searching for novel natural compounds to replace chemical preservatives, as the alternatives are presently quite limited[5].

Chitosan, which is derived from the partial deacetylation of chitin under alkaline conditions, is a biopolymer which has been found to be useful for applications in a wide range of fields[6,7]. It has unique physiochemical properties and biological activities, which have aroused interest in it as a natural material for use in cosmetics[8]. Specifically, chitosan has antimicrobial[9,10], antioxidant[11], wound healing[12], and pharmaceutical activities[13] and is non-toxic[14]. Its properties are affected by degree of deacetylation, molecular weight, and positive charge content, as well as the temperature and pH of the reaction conditions[15]. Chitosan has been attracting increased interest in the cosmetic industry and has been used as a skin protecting and emulsifying agent, antimicrobial, antioxidant, and as a delivery system[16,17].

The antimicrobial properties of natural materials are generally affected by reaction and combination conditions. For this reason, the antimicrobial activity of natural materials depends on both the medium and the specific formulations in which they are embedded[18]. Therefore, this study aimed to investigate the properties of chitosan in cosmetic formulations by testing the synergistic or antagonistic antibacterial and antioxidant activities of different molecular weight chitosans with common cosmetic ingredients.

2. Materials and Methods

2.1 Chemicals and materials

Chitosan of different molecular weights was used for experiments. High molecular weight chitosan (HMWC, 600 kDa) was purchased from Sokchomulsan Co. Ltd (Sokchomulsan, Korea). Medium molecular weight chitosan (MMWC, 300 kDa) and low molecular weight chitosan (LMWC, 0.8-3.0 kDa) used for experiments were prepared as described in a previous study[10]. Lactic acid used for the preparation of chitosan solution as a solvent, was purchased from Sigma Chemical Co. (Sigma Chemical Co., USA). Cosmetic ingredients (1,2-hexanediol, 1,3-butylene glycol, EDTA-2Na, ethanol, and glycerol) were purchased from Sigma.

2.2 Bacteria Culture

Bacterial strains used for experiments were Gram-positive bacteria, *Staphylococcus aureus* (*S. aureus*, ATCC 29213) and methicillin-resistant *Staphylococcus aureus* (MRSA), and a Gram-negative bacterium *Escherichia coli* (*E. coli*). *S. aureus* was purchased from the Korea Culture Center of Microorganisms (KCCM, Korea). MRSA was isolated from Gachon University Gil Hospital (Gachon University Gil Hospital, Korea) and stored in a −80 ℃ freezer until further use[19]. *E. coli* was isolated from commercially available bought pork and confirmed with 16S rRNA sequencing[20]. The strains were streaked onto LB (Luria-Bertani)

agar and incubated at 37 ℃ for 24 h. After that, single colonies were incubated in the medium in a shaking incubator for 24 h.

2.3 Disc diffusion assay

Each bacterial strain (1 x 10^8 CFU/mL) pre-cultured in LB medium was mixed at a ratio of 1 mL in 100 mL of liquid medium containing 1.5 % (w/v) agar, and the medium containing bacteria was poured into disposal petri-dishes. While the media solidified, 100 μL of samples were inoculated onto paper-discs (0.8 cm/diameter), which were then placed carefully on the solidified agar media. After confirming the discs attached to the media, the plates were incubated at 37 ℃ for 24 h. Time after, the diameter of the clear zones was measured and recorded.

2.4 Minimum Inhibitory Concentration

The initial bacterial cell number was adjusted to be 5 x 10^5 CFU/mL in the medium. Pre-cultured 100 μL of bacterial cells were inoculated to wells of 96-well plates. Then, 100 μL samples were added to each well. The absorbance was monitored by spectrophotometer at 595 nm (Multiskan FC, Thermo Fisher Scientific, USA) and the plates were cultured continuously for up to 24 h at 37 ℃. Minimum inhibitory concentration (MIC) was defined as the lowest concentration with no growth.

2.5 Checkerboard synergy assay

Synergistic antibacterial activity of chitosan with cosmetic ingredients was evaluated by checkerboard synergy assay to obtain the fractional inhibitory concentration (FIC). The first compound was horizontally serially diluted, while another compound was vertically serially diluted. Each compound was aliquotted at 100 μL in wells of 96-well plates. Then, 100 μL of medium containing bacteria (5 x 10^5 CFU/mL) was added to the wells of plates. The absorbance was monitored by spectrophotometer at 595 nm and the plates were cultured continuously for up to 24 h at 37 ℃. FIC index was calculated using the following equation (1).

$$FIC_{index} = FIC_A + FIC_B \tag{1}$$

Where, FIC_A denotes MIC of compound A in combination / MIC of compound A alone, FIC_B denotes MIC of compound B in combination / MIC of compound B alone

The results were interpreted as synergistic (FIC index ≤ 0.5), additive or no interaction (0.5 〈 FIC index ≤ 1.0), or antagonistic (FIC index 〉 1.0).

2.6 DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay

100 μL of samples were aliquotted to wells of 96-well plates that had 100 μL 0.1 mM DPPH (dissolved in ethanol) added. Ascorbic acid was used as a positive-control. The plates were stored in a darkroom at 25 ℃ for 30 min. The absorbance was measured at 595 nm using a spectrophotometer. DPPH radical scavenging activity was calculated using the following equation (2).

$$DPPH\ radical\ scavenging\ activity\ (\%) = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100 \tag{2}$$

Where, $Abs_{control}$ denotes absorbance of control, Abs_{sample} denotes absorbance of samples

2.7 Statistical analysis

The results from three independent experiments are presented in this study as the means of average values. Average values were calculated as means and standard errors (± SE). The statistical significance of differences was

assessed by the analysis of variance (ANOVA) with significance assumed at $p < 0.05$.

3. Results and Discussion

3.1 Disc diffusion assay

Disc diffusion assays were performed to evaluate the antibacterial activity of chitosan of different molecular weights. The antibacterial activity of chitosan was seen to be dependent on both its molecular weight and concentration (Table 1). HMWC and MMWC exhibited no antibacterial activity at a concentration of 625 μg/mL, while LMWC inhibited bacterial growth at this concentration, with clear zone diameters of (9.07±0.09) and (9.20±0.15) mm. Distilled water had no antibacterial activity.

Earlier studies, also showed that the antibacterial activity of chitosan was dependent on the molecular weight and that much greater antibacterial activity was observed with lower molecular weight chitosan[21,22]. Our data are consistent with this and demonstrate that in addition to lower molecular weight chitosan having higher antibacterial activity, the effect was dose-dependent.

Table 1. Diameter of the clear zone of different molecular weight chitosan toward *S. aureus*, MRSA, *E. coli*

Strains[2]	Chitosan[3]	Diameter of the clear zone (mm)[1]					
		Distilled Water	Chitosan concentration (μg/mL)				
			5000	2500	1250	625	312.5
S. aureus	HMWC	NA[4]	11.93±0.32	10.67±0.03	8.83±0.24	NA	NA
	MMWC	NA	11.97±0.15	10.50±0.21	9.07±0.15	NA	NA
	LMWC	NA	18.90±0.12	16.20±0.60	12.33±0.23	9.07±0.09	NA
MRSA	HMWC	NA	11.73±0.35	10.27±0.18	8.63±0.09	NA	NA
	MMWC	NA	11.67±0.24	10.10±0.06	8.77±0.15	NA	NA
	LMWC	NA	18.67±0.24	16.27±0.37	12.67±0.41	9.20±0.15	NA
E. coli	HMWC	NA	11.93±0.13	10.30±0.21	8.83±0.23	NA	NA
	MMWC	NA	11.20±0.21	10.03±0.12	8.50±0.25	NA	NA
	LMWC	NA	17.57±0.23	13.40±0.31	10.27±0.18	9.10±0.12	NA

[1] All values represent the average diameter (mean ± SE) of triplicate tests, with $p < 0.05$ indicating statistical significance
[2] *S. aureus*: *Staphylococcus aureus*; MRSA: methicillin-resistant *Staphylococcus aureus*; *E. coli*: *Escherichia coli*
[3] HMWC: high molecular weight chitosan; MMWC: middle molecular weight chitosan; LMWC: low molecular weight chitosan
[4] NA means as no activity

Table 2. MIC (%) of different molecular weight chitosan and cosmetic ingredients toward *S. aureus*, MRSA, *E. coli*

Samples		Strains[1]		
		S. aureus	MRSA	*E. coli*
Chitosan[2]	HMWC	0.125	0.125	0.125
	MMWC	0.125	0.125	0.125
	LMWC	0.0312	0.0312	0.0312
Cosmetic ingredients	1,2-hexanediol	2	2	2
	1,3-butylene glycol	20	20	20
	EDTA-2Na[3]	0.0156	0.0156	0.0078
	Ethanol	25	25	25
	Glycerol	40	40	40

[1] *S. aureus*: *Staphylococcus aureus*; MRSA: methicillin-resistant *Staphylococcus aureus*; *E. coli*: *Escherichia coli*
[2] HMWC: high molecular weight chitosan; MMWC: middle molecular weight chitosan; LMWC: low molecular weight chitosan
[3] Ethylenediaminetetraacetic acid disodium salt

Table 3. Synergistic antibacterial activity of different molecular weight chitosan with cosmetic ingredients toward *S .aureus*, MRSA, *E. coli*

Strains[1]	Compound[2]		FIC[3] value		FIC index	Interpretation
	A	B	FIC_A	FIC_B		
S. aureus	HMWC	1,2-hexanediol	0.5	0.25	0.75	Additive
		1,3-butylene glycol	0.5	0.5	1	Additive
		EDTA-2Na	0.25	0.25	0.5	Synergistic
		Ethanol	0.125	0.125	0.25	Synergistic
		Glycerol	0.25	0.25	0.5	Synergistic
	MMWC	1,2-hexanediol	0.5	0.25	0.75	Additive
		1,3-butylene glycol	0.5	0.5	1	Additive
		EDTA-2Na	0.5	0.125	0.625	Additive
		Ethanol	0.25	0.25	0.5	Synergistic
		Glycerol	0.25	0.25	0.5	Synergistic
	LMWC	1,2-hexanediol	0.5	0.125	0.625	Additive
		1,3-butylene glycol	0.25	0.5	0.75	Additive
		EDTA-2Na	0.25	0.125	0.375	Synergistic
		Ethanol	0.25	0.25	0.5	Synergistic
		Glycerol	0.25	0.25	0.5	Synergistic
MRSA	HMWC	1,2-hexanediol	0.5	0.25	0.75	Additive
		1,3-butylene glycol	0.5	0.5	1	Additive
		EDTA-2Na	0.25	0.25	0.5	Synergistic
		Ethanol	0.125	0.125	0.25	Synergistic
		Glycerol	0.25	0.25	0.5	Synergistic
	MMWC	1,2-hexanediol	0.5	0.25	0.75	Additive
		1,3-butylene glycol	0.5	0.5	1	Additive
		EDTA-2Na	0.5	0.125	0.625	Additive
		Ethanol	0.25	0.25	0.5	Synergistic
		Glycerol	0.25	0.25	0.5	Synergistic
	LMWC	1,2-hexanediol	0.5	0.125	0.625	Additive
		1,3-butylene glycol	0.25	0.5	0.75	Additive
		EDTA-2Na	0.25	0.125	0.375	Synergistic
		Ethanol	0.25	0.25	0.5	Synergistic
		Glycerol	0.25	0.25	0.5	Synergistic
E. coli	HMWC	1,2-hexanediol	0.5	0.125	0.625	Additive
		1,3-butylene glycol	0.5	0.5	1	Additive
		EDTA-2Na	0.25	0.125	0.375	Synergistic
		Ethanol	0.125	0.125	0.25	Synergistic
		Glycerol	0.25	0.25	0.5	Synergistic
	MMWC	1,2-hexanediol	0.5	0.25	0.75	Additive
		1,3-butylene glycol	0.5	0.5	1	Additive
		EDTA-2Na	0.5	0.125	0.625	Additive
		Ethanol	0.25	0.25	0.5	Synergistic
		Glycerol	0.25	0.25	0.5	Synergistic
	LMWC	1,2-hexanediol	0.5	0.125	0.625	Additive
		1,3-butylene glycol	0.25	0.5	0.75	Additive
		EDTA-2Na	0.25	0.125	0.375	Synergistic
		Ethanol	0.25	0.125	0.375	Synergistic
		Glycerol	0.25	0.25	0.5	Synergistic

[1] *S. aureus*: *Staphylococcus aureus*; MRSA: methicillin-resistant *Staphylococcus aureus*; *E. coli*: *Escherichia coli*
[2] HMWC: high molecular weight chitosan; MMWC: middle molecular weight chitosan; LMWC: low molecular weight chitosan; EDTA-2Na: ethylenediaminetetraacetic acid disodium salt
[3] Fractional inhibitory concentration

3.2 Minimum Inhibitory Concentration

The MICs of chitosan with different molecular weights and cosmetic ingredients were determined by broth microdilution method. Results are shown in Table 2. LMWC showed a remarkable antibacterial activity with 4-fold lower MIC than HMWC and MMWC. The antibacterial activities of EDTA-2Na were slightly different toward Gram-positive and Gram-negative bacteria. The MIC of EDTA-2Na was 2-fold lower in Gram-negative compared with Gram-positive bacteria. EDTA-2Na revealed significant antibacterial activity with the lowest MIC.

Chitosan of lower molecular weight showed the highest antibacterial activity and in our hands, EDTA-2Na showed stronger antibacterial activities toward Gram-negative bacteria. However, previous studies indicated that EDTA is more potent at growth inhibition in Gram-positive than Gram-negative bacteria[23,24].

3.3 Checkerboard synergy assay

To evaluate the synergistic antibacterial activities of chitosan with different cosmetic ingredients, checkerboard synergy assays were performed. Chitosan showed synergistic antibacterial activity particularly in the presence of EDTA-2Na and ethanol which each showed remarkable synergistic effects toward both Gram-positive and Gram-negative bacteria (Table 3). *Kim et al.*[18] stated that EDTA-2Na showed MIC of 0.0625 % and 2 % in Gram-positive and Gram-negative bacteria, respectively. In our hands, EDTA-2Na with LMWC revealed MIC of 0.0019 % and 0.0098 % in Gram-positive and Gram-negative bacteria, respectively. Ethanol showed MIC of 25 % in Gram-positive and Gram-negative bacteria[18]. Ethanol with LMWC showed inhibition of bacterial growth by MICs that were reduced (6.25 % and 3.12 %) in Gram-positive and Gram-negative bacteria.

The antibacterial mechanism of chitosan was suggested that its positive charge of the amino group combines with anionic components on the cell membrane. Moreover, chitosan inhibits bacterial growth by chelating transition metal ions[25]. Earlier studies stated that EDTA inhibits microbial growth by eliminating Mg^{2+} and Ca^{2+} ions of the outer cell wall of Gram-negative bacteria and by reducing iron absorption. In addition, the iron-chelating property of EDTA may enhance the efficacy of other antimicrobial agents[24,26]. For these reasons, synergistic interaction between chitosan and EDTA-2Na may enhance their antibacterial activity. Our results showed that chitosan revealed potent antibacterial synergistic effects with EDTA-2Na in Gram-negative bacteria. Ethanol inhibits the biosynthesis of peptidoglycan and affects the outer membrane of Gram-negative bacteria[27,28]. Chitosan and ethanol exhibit antibacterial activities by disrupting the outer membrane of Gram-negative bacteria. Similar antibacterial mechanisms of chitosan and ethanol on the outer membrane may cause the synergistic antibacterial effects. The additive effects of chitosan with some cosmetic ingredients are considered to be due to independent antibacterial mechanisms.

The checkerboard synergy assay revealed that in order from stronger to weaker, synergistic antibacterial effects were bestowed by LMWC, HMWC, and then MMWC. Chitosan with cosmetic ingredients showed no antagonistic effect. Therefore, chitosan would be expected to facilitate potent antibacterial activity in cosmetic formulations.

3.4 DPPH radical scavenging assay

The DPPH radical scavenging activity of different chitosans and cosmetic ingredients is shown in Fig. 1 and was found to be; HMWC (21.7±0.1 %), MMWC (23.2±0.7 %), and LMWC (57.3±0.6 %). The radical scavenging activity of cosmetic ingredients were: 1,2-hexanediol (3.2±0.1 %),

Table 4. IC_{50} value of DPPH radical scavenging activity from different molecular weight chitosan

IC_{50} (mg/mL)	HMWC	MMWC	LMWC
DPPH	0.65±0.01	0.60±0.02	0.12±0.00

* All values represent the means of triplicate tests and standard error (± SE), with $p < 0.05$ indicating statistical significance
* IC_{50}: the half-maximal inhibitory concentration; HMWC: high molecular weight chitosan; MMWC: middle molecular weight chitosan; LMWC: low molecular weight chitosan; DPPH: 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay

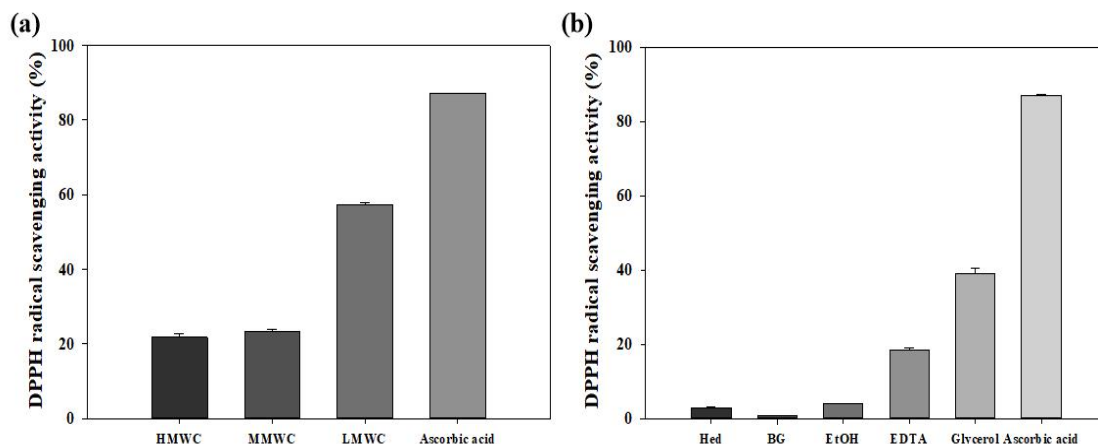

Fig. 1. DPPH radical scavenging activity of different molecular weight chitosan and cosmetic ingredients.
(a) different molecular weight chitosan (1250 μg/mL) (b) cosmetic ingredients (MIC). HMWC: high molecular weight chitosan; MMWC: middle molecular weight chitosan; LMWC: low molecular weight chitosan; Hed: 1,2-hexanediol; BG: 1,3-butylene glycol; EtOH: ethanol; EDTA: ethylenediaminetetraacetic acid disodium salt. Ascorbic acid (1250 μg/mL) was used as a positive-control. All values represent the means of triplicate tests and error bars represent standard error (± SE), with $p < 0.05$ indicating statistical significance.

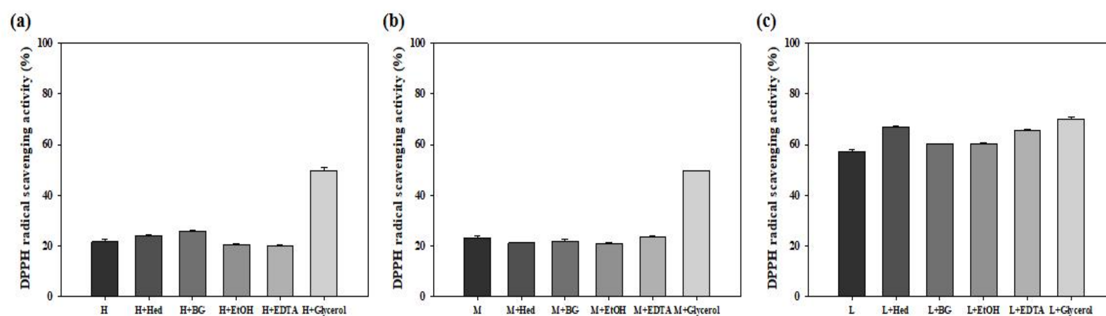

Fig. 2. DPPH radical scavenging activity of different molecular weight chitosan with cosmetic ingredients.
(a) HMWC with cosmetic ingredients (b) MMWC with cosmetic ingredients (c) LMWC with cosmetic ingredients. H: high molecular weight chitosan; M: middle molecular weight chiotsan; L: low molecular weight chitosan; Hed: 1,2-hexanediol; BG: 1,3-butylene glycol; EtOH: ethanol; EDTA: ethylenediaminetetraacetic acid disodium salt. All values represent the means of triplicate tests and error bars represent standard error (± SE), with $p < 0.05$ indicating statistical significance.

1,3-butylene glycol (0.7±0.0 %), ethanol (3.9±0.0 %), EDTA-2Na (18.5±0.4 %), and glycerol (39.0±1.4 %). Ascorbic acid, positive-control, showed a radical scavenging activity of 87.0±0.1 %.

The DPPH radical scavenging activity of different chitosans with cosmetic ingredients is shown in Fig. 2 and was found to be: HMWC+1,2-hexanediol (23.9±0.8 %), HMWC+1,3-butylene glycol (25.9±0.5 %), HMWC+ethanol (20.4±0.0 %), HMWC+ EDTA-2Na (19.9±0.5 %), and HMWC+glycerol (49.8±1.0 %); MMWC+1,2-hexanediol (21.1±0.4 %), MMWC+1,3-butylene glycol (22.0±0.7 %), MMWC+ethanol (20.9±0.6 %), MMWC+EDTA-2Na (23.5±0.4 %), and MMWC+glycerol (49.6±0.0 %); LMWC+1,2-hexanediol (67.0±0.1 %), LMWC+ 1,3-butylene glycol (60.3±0.1 %), LMWC+ethanol (60.4±0.5 %), LMWC+EDTA-2Na (65.6±0.1 %), LMWC+glycerol (70.1±0.7 %).

The half-maximal inhibitory concentration (IC_{50}) of DPPH radical scavenging activity was much lower in LMWC, compared to HMWC and MMWC (Table 4).

LMWC showed increasing antioxidant activities with all of the ingredients (Fig. 2). HMWC and MMWC showed additive effects with cosmetic ingredients. Chang *et al.*[29] stated that the molecular weight of chitosan is a crucial factor of its antioxidant properties, along with the degree of deacetylation. In addition, other earlier studies found that the antioxidant effects of chitosan increased as molecular weight decreased[29,30]. Similarly, we confirmed that the antioxidant activities of chitosan were inversely correlated with its molecular weight.

4. Conclusion

In this study, we confirmed synergistic effects of chitosans in combination with cosmetic ingredients, on antibacterial and antioxidant activities. In particular, the results showed significant antibacterial and antioxidant activities exhibited by LMWC. EDTA-2Na and ethanol showed robust synergistic antibacterial effects and revealed stronger efficacy toward Gram-negative bacteria. Combinations of LMWC and EDTA-2Na or ethanol, should therefore be strongly considered as preservative options in cosmetic formulations susceptible to Gram-negative bacteria. Our data suggest that chitosan has potent properties as a natural material for use in cosmetic formulations and may be useful in a wide range of applications in the cosmetic industry.

References

[1] N. Halla, I. P. Fernandes, S. A. Heleno, P. Costa, Z. Boucherit-Otmani, K. Boucherit, A. E. Rodrigues, I. C. F. R. Ferreira, M. F. Barreiro, "Cosmetics Preservation: A Review on Present Strategies", *Molecules*, Vol.23, No.7, pp.1571, 2018.
DOI: https://doi.org/10.3390/molecules23071571

[2] A. Yorgancioglu, E. E. Bayramoglu, "Production of cosmetic purpose collagen containing antimicrobial emulsion with certain essential oils", *Ind Crops Prod*, Vol.44, pp.378-382, 2013.
DOI: https://doi.org/10.1016/j.indcrop.2012.11.013

[3] J. Wang, Y. Liu, W. R. Kam, Y. Li, D. A. Sullivan, "Toxicity of the cosmetic preservatives parabens, phenoxyethanol and chlorphenesin on human meibomian gland epithelial cells", *Exp. Eye Res.*, Vol.196, 2020.
DOI: https://doi.org/10.1016/j.exer.2020.108057

[4] E. Y. Lee, A. Susan, D. W. Choi, S. J. Moon, I. S. Chang, "Comparison of objective and sensory skin irritations of several cosmetic preservatives", *Contact Derm.*, Vol.56, No.3, pp.131-136, 2007.
DOI: https://doi.org/10.1111/j.1600-0536.2007.01001.x

[5] A. Kerdudo, P. Burger, F. Merck, A. Dingas, Y. Rolland, T. Michel, X. Fernandez, "Development of a natural ingredient - Natural preservative: A case study", *C R Chim*, Vol.19, No.9, pp.1077-1089, 2016.
DOI: https://doi.org/10.1016/j.crci.2016.06.004

[6] W. Tan, J. Zhang, F. Luan, L. Wei, Y. Chen, F. Dong, Q. Li, Z. Guo, "Design, synthesis of novel chitosan derivatives bearing quaternary phosphonium salts and evaluation antifungal activity", *Int. J. Biol. Macromol.*, Vol.102, pp.704-711, 2017.
DOI: https://doi.org/10.1016/j.ijbiomac.2017.04.073

[7] S. Sharma, A. Kumar, R. Kumar, N. K. Rana, B. Koch, "Development of a novel chitosan based

biocompatible and self-healing hydrogel for controlled release of hydrophilic drug", *Int. J. Biol. Macromol.*, Vol.116, pp.37-44, 2018.
DOI: https://doi.org/10.1016/j.ijbiomac.2018.05.020

[8] Z. Guo, R. Xing, S. Liu, Z. Zhong, X. Ji, L. Wang, P. Li, "Antifungal properties of Schiff bases of chitosan, N-substituted chitosan and quaternized chitosan", *Carbohydr. Res.*, Vol.342, No.10, pp.1329-1332, 2007.
DOI: https://doi.org/10.1016/j.carres.2007.04.006

[9] G. Y. Nam, D. G. Lee, J. M. Kim, Y. J. Hwang, T. H. Song, J. K. Park, "Effect of Active Molecular Chitosan with Antifungal Activity on the Quality Properties of Tuna Extract", *JCC*, Vol.23, No.1, pp.29-35, 2018.
DOI: https://doi.org/10.17642/jcc.23.1.5

[10] J. W. Kim, J. K. Park, "Synergistic antimicrobial properties of active molecular chitosan with EDTA-divalent metal ion compounds", *J Phytopathol*, Vol.165, No.10, pp.641-651, 2017.
DOI: https://doi.org/10.1111/jph.12603

[11] Y. H. Lee, S. Y. Park, J. E. Park, B. O. Jung, J. E. Park, J. K. Park, Y. J. Hwang, "Anti-Oxidant Activity and Dust-Proof Effect of Chitosan with Different Molecular Weights", *Int. J. Mol. Sci.*, Vol.20, No.12, pp.3085, 2019.
DOI: https://doi.org/10.3390/ijms20123085

[12] M. Abbas, T. Hussain, M. Arshad, A. R. Ansari, A. Irshad, J. Nisar, F. Hussain, N. Masood, A. Nazir, M. Iqbal, "Wound healing potential of curcumin cross-linked chitosan/polyvinyl alcohol", *Int. J. Biol. Macromol.*, Vol.140, pp.871-876, 2019.
DOI: https://doi.org/10.1016/j.ijbiomac.2019.08.153

[13] D. Raafat, K. Von Bargen, A. Haas, H. G. Sahi, "Insights into the Mode of Action of Chitosan as an Antibacterial Compound", *Appl. Environ. Microbiol.*, Vol.74, No.12, pp.3764-3773, 2008.
DOI: https://doi.org/10.1128/AEM.00453-08

[14] M. Thanou, J. C. Verhoef, H. E. Junginger, "Oral drug absorption enhancement by chitosan and its derivatives", *Adv. Drug Deliv. Rev.*, Vol.52, No.2, pp.117-126, 2001.
DOI: https://doi.org/10.1016/S0169-409X(01)00231-9

[15] S. H. Chang, H. T. V. Lin, G. J. Wu, G. J. Tsai, "pH Effects on solubility, zeta potential, and correlation between antibacterial activity and molecular weight of chitosan", *Carbohydr. Polym.*, Vol.134, pp.74-81, 2015.
DOI: https://doi.org/10.1016/j.carbpol.2015.07.072

[16] I. Aranaz, N. Acosta, C. Civera, B. Elorza, J. Mingo, C. Castro, M. De los Lianos Gandia, A. Heras Caballero, "Cosmetics and Cosmeceutical Applications of Chitin, Chitosan and Their Derivatives", *Polymers*, Vol.10, No.2, pp.213, 2018.
DOI: https://doi.org/10.3390/polym10020213

[17] I. G. Jung, G. M. Lee, Y. J. Hwang, "Preservative Effect of Chitosan with 1,3-Butylene Glycol as Natural Preservative in Cosmetic Formulation", *JCC*, Vol.26, No.2, pp.69-75, 2021.
DOI: https://doi.org/10.17642/jcc.26.2.0

[18] S. H. Kim, Y. N. Yun, S. S. An, "A Study on Synergistic Antibacterial Activity of Cosmetic Ingredients", *J. Soc. Cosmet. Scientists Korea*, Vol.44, No.1, pp.1-7, 2018.
DOI: https://doi.org/10.15230/SCSK.2018.44.1.1

[19] Y. S. Mun, Y. J. Hwang, "Novel *spa* and Multi-Locus Sequence Types (MLST) of *Staphylococcus Aureus* Samples Isolated from Clinical Specimens in Korean", *Antibiotics*, Vol.8, No.4, pp.202, 2019.
DOI: https://doi.org/10.3390/antibiotics8040202

[20] Y. J. Hwang, "Molecular detection of bla_{VIM}, bla_{BIC}, bla_{KPC}, and bla_{SIM} genes from isolated bacteria in retail meats", *JKAIS*, Vol.22, No.6, pp.413-419, 2021.
DOI: https://doi.org/10.5762/KAIS.2021.22.6.413

[21] R. C. Goy, D. D. Britto, O. B. G. Assis, "A review of the antimicrobial activity of chitosan", *Polimeros*, Vol.19, No.3, pp.241-247, 2009.
DOI: https://doi.org/10.1590/S0104-14282009000300013

[22] N. Liu, X. G. Chen, H. J. Park, C. G. Liu, C. S. Liu, X. H. Meng, L. J. Yu, "Effect of MW and concentration of chitosan on antibacterial activity of *Escherichia coli*", *Carbohydr. Polym.*, Vol.64, No.1, pp.60-65, 2006.
DOI: https://doi.org/10.1016/j.carbpol.2005.10.028

[23] J. Kong, Z. X. Wu, L. Wei, Z. S. Chen, S. Yoganathan, "Exploration of Antibiotic Activity of Aminoglycosides, in Particular Ribostamycin Alone and in Combination With Ethylenediaminetetraacetic Acid Against Pathogenic Bacteria", *Front. Microbiol.*, Vol.11, pp.1718, 2020.
DOI: https://doi.org/10.3389/fmicb.2020.01718

[24] D. H. Qiu, Z. L. Huang, T. Zhou, C. Shen, and R. C. Hider, "*In vitro* inhibition of bacterial growth by iron chelators", *FEMS Microbiol. Lett.*, Vol.314, No.2, pp.107-111, 2010.
DOI: https://doi.org/10.1111/j.1574-6968.2010.02153.x

[25] A. A. El-Sharif, M. H. M. Hussan, "Chitosan–EDTA New Combination is a Promising Candidate for Treatment of Bacterial and Fungal Infections", *Curr. Microbiol.*, Vol.62, No.3, pp.739-745, 2011.
DOI: https://doi.org/10.1007/s00284-010-9777-0

[26] S. Finnegan, S. L. Percival, "EDTA: An Antimicrobial and Antibiofilm Agent for Use in Wound Care", *Adv. Wound Caref*, Vol.4, No.7, pp.415-421, 2015.
DOI: https://doi.org/10.1089/wound.2014.0577

[27] B. Soufi, K. Krug, A. Harst, B. Macek, "Characterization of the *E. coli* proteome and its modifications during growth and ethanol stress", *Front. Microbiol.*, Vol.6, No.103, 2015.
DOI: https://doi.org/10.3389/fmicb.2015.00103

[28] K. M. Lupino, K. A. Romano, M. J. Simons, J. T. Gregg, L. Panepinto, G. M. Gruz, L. Grajek, G. A. Caputo, M. J. Hickman, G. B. Hecht, "A Recurrent Silent Mutation Implicates *fecA* in Ethanol Tolerance by *Escherichia coli*", *BMC Microbiol*, Vol.18, No.1, pp.1-17, 2018.

DOI: https://doi.org/10.1186/s12866-018-1180-1

[29] S. H. Chang, C. H. Wu, G. J. Tsai, "Effects of chitosan molecular weight on its antioxidant and antimutagenic properties", *Carbohydr. Polym.*, Vol.181, pp.1026-1032, 2018.
DOI: https://doi.org/10.1016/j.carbpol.2017.11.047

[30] H. Tomida, T. Fujii, N. Furutani, A. Michihara, T. Yasufuku, K. Akasaki, T. Maruyama, M. Otagiri, J. M. Gebicki, M. Anraku, "Antioxidant properties of some different molecular weight chitosans", *Carbohydr. Res.*, Vol.344, No.13, pp.1690-1696, 2009.
DOI: https://doi.org/10.1016/j.carres.2009.05.006

In Geun Jung [Associate member]

• Mar. 2019 ~ current : Gachon University, Dept. of Biomedical Engineering.

〈Research Interests〉
Molecular Biology, Functional Natural Substances.

Guen Moo Lee [Associate member]

• Mar. 2016 ~ current : Gachon University, Dept. of Biomedical Engineering.

〈Research Interests〉
In Vitro Diagnostic Medical Device, Regulatory Affairs.

You Jin Hwang [Life member]

• Feb. 1990 : Inha University MS.
• Feb. 1999 : Inha University PhD.
• Mar. 2000 ~ 2014 : Gachon University, Dept. of Biological Science.
• Feb. 2015 ~ current : Gachon University, Dept. of Biomedical Engineering Professor.

〈Research Interests〉
Cancer & Microenvironment Molecular Imaging, Functional Natural Substances.