Vol. 16(12) pp. 334-342, December 2022
DOI: 10.5897/AJFS2022.2231
Article Number: F73131070088
ISSN: 1996-0794

http://www.academicjournals.org/AJFS

African Journal of Food Science

Full Length Research Paper

Increased antibacterial activity of *Angelica koreana* and *Arnebia euchroma* extracts fermented by *Lactobacillus acidifarinae* against methicillin-resistant *Staphylococcus aureus*

Jae-Goo Kim[1], Yunji Cha[2], Seung-Bo Yang[3], Jiyoung Kim[2] and Ki-Young Kim[1*]

[1]Graduate School of Biotechnology, Kyung Hee University, Gyeonggi-do, Republic of Korea.
[2]Cosmogen Co., Ltd. Suwon-si, Gyeonggi-do, Republic of Korea.
[3]Department of Korean Internal Medicine, College of Korean Medicine, Gachon University, Seongnam-si, Gyeonggi-do, Republic of Korea.

Received 18 October, 2022; Accepted December 7, 2022

The increasing epidemic of methicillin-resistant *Staphylococcus aureus* (MRSA), one of the most important hospital and community pathogens, has led to a demand for new agents to treat the infection. Natural products may be used to reduce this problem with low side effects. The objective of this study was to determine the antibacterial effect of fermented extracts of *Angelica koreana* and *Arnebia euchroma* by *Lactobacillus* spp. against *S. aureus*, which were tested by disk diffusion test. Extracts of *A. koreana* and *A. euchroma* showed a clear zone of 15.5 ± 1.1 and 15.9 ± 2.2 mm, respectively. Fermented extracts by *Lactobacillus* sp. showed more improved antibacterial activity against *S. aureus* than the extracts.

Key words: MRSA; *Lactobacillus* spp.; fermentation; disk diffusion test; plant extract.

INTRODUCTION

Staphylococcus aureus is a major pathogen causing nosocomial infections. The emergence of antibiotic-resistant strains of *S. aureus* that caused infections among hospitalized patients is a severe problem worldwide (Li and Webster, 2018).

For example, the rate of hospital-acquired MRSA reached 50.4% in China and MRSA is causative of almost 44% of cases and over 20% of excess mortality among healthcare-acquired infections in Europe (Guo et al., 2020). Treatment options for MRSA infection are currently limited because most MRSA strains are resistant to widely used antibiotics such as lactams, macrolides, aminoglycosides, and fluoroquinolones (Schentag et al., 1998; Tacconelli et al., 2008; Kaur and Chate, 2015). Therefore, it is necessary to find alternative treatments to prevent and control MRSA infections.

Angelica koreana, also named *Ostericum koreanum*, has traditionally been used in oriental Korean medicine to

*Corresponding author. E-mail: kiyoung@khu.ac.kr.

treat the common cold, and to reduce rheumatic pains or headaches. This plant has been reported for various biological activities including anti-tumor, anti-microbial, antioxidant, and anti-inflammatory effects (Kang et al., 2009; Shin, 2005; Park et al., 2007, 2008).

Arnebia euchroma Royle is a well-known traditional herb used for various skin diseases in Iranian tribal medicine (Ashkani-Esfahani et al., 2012). Shikonin derivatives isolated from the roots of *A. euchroma* have been reported for antimicrobial, anti-inflammatory and anti-tumor activities and thus to be considered as important compounds for potential medicinal use (Kim et al., 2001).

The bioconversion process could enhance the biological activities of medicinal plants and herbs. A previous study showed that lactic fermented herbal teas have more composition in phenolic, flavonoid compounds (Ibrahim et al., 2014). Another study also showed that the antioxidant and antibacterial activities of medicinal plants fermented by fungi are increased compared to non-fermented control (Dong et al., 2015). *Lactobacillus*-fermented *Artemisia princeps* has been used as a functional component to increase the growth performance, meat lipid stability, and intestinal health of chickens (Kim et al., 2012).

This study aimed to evaluate the antibacterial effects of *A. koreana* and *A. euchroma* extracts(AK and AE, respectively) after fermentation by *Lactobacillus* spp. against *S. aureus* using the disk diffusion and biofilm formation method.

MATERIALS AND METHODS

Raw materials

The roots of *A. koreana* and *A. euchroma* Royle were commercially purchased (Barumhanyak, Korea). 1 kg each of dried plant roots was immersed in 5-10 volumes of 70-80% ethanol. They were extracted by maceration for overnight with constant shaking at 24 °C 3 times. The obtained extracts were filtered with Whatman 2 filter paper to discard impurities. The filtered extract was concentrated under reduced pressure in a rotary concentrator and dried to obtain a solid content of the extract.

Bacterial strains and culture

S. aureus (CCARM3505, MRSA and CCARM3506, QRSA), *Lactobacillus acidophilus* (KACC12419, AD), *Lactobacillus acidifarinae* (KACC16342, AF) and *Lactobacillus acidipiscis* (KCTC12394, AP) were purchased from CCARM (The Canadian Centre for Agri-Food Research in Health and Medicine) and KCTC (Korean Collection for Type Cultures). All strains were kept in 20% glycerol at -70°C. *S. aureus* was cultured in tryptic soy broth (TSB, BD Difco, Franklin Lakes, USA) containing tryptone 17 g, soytone 3 g, glucose 2.5 g, sodium chloride 5 g, and dipotassium phosphate 2.5 g at 37°C for 24 h. *Lactobacillus* spp. were cultured in MRS containing Lactobacilli MRS Broth 55 g/L (BD Difco, Franklin Lakes, USA) at 37 °C for 24 hours under anaerobic condition (Bae et al., 2019).

Fermentation of *A. koreana* and *A. euchroma* with *Lactobacillus* spp.

Extracts of *A. koreana* and *A. euchroma* were inoculated to be 1% of the total volume at 10^7 CFU/mL of *Lactobacillus* spp. Fermentation was carried out in 14 mL round tube for 24-96 h at 37°C under anaerobic condition. Filtration of the supernatant was done using a nominal 0.22 µm filter (ProLabs, Korea) to remove residual cells (Hashemi et al., 2017). It was repeated three or more times to produce fermentations.

Antimicrobial susceptibility testing

Bacterial suspensions with a turbidity equivalent to a McFarland standard of 0.5 were swabbed evenly onto TSA plates with a sterile cotton swab for the disk diffusion method. Antibiotic disks containing ampicillin, plant extracts, and fermented extracts were placed on TSA plates. The plates were incubated at 37 °C for 24 h and then the inhibition zone diameters, including the diameter of the disk, were measured (Dušková and Karpíšková, 2013). The test was repeated three or more times and all data are the average ± STDEV.

Biofilm formation assay

A slightly modified biofilm formation assay was used (Hobby et al., 2012; O'Toole, 2011). The 6-well plate was incubated with extracts at 37°C for 24 h; the wells were gently washed twice with 200 µL of phosphate-buffered saline (PBS) to remove all planktonic cells. After aspiration of planktonic cells, adherent biofilms were fixed with absolute ethanol before staining with 200 µL 0.2% crystal violet solution (Thermo Fisher Scientific, USA) for 5 min. Following aspiration of the stain, wells were washed three times with PBS and air-dried. A quantitative assessment of biofilm formation was then taken by adding 33% acetic acid and incubating for 10 min. The absorbance of eluate was calculated from optical density (OD_{570}) values measured using a microplate reader (BioTek Instruments, Korea). The assay was repeated three or more times and all data are the average ± STDEV.

Cell viability assay

A slightly modified MTT assay was used to test the cell viability of HaCaT cells (Park et al., 2021). Briefly, HaCaT cells in Dulbecco's modified eagle's medium (DMEM) at a density of 10^4 cells are cultured in a 96-well plate for 24 h. Serum-free medium containing extracts or fermented extracts was added to the wells. After 24 h of incubation, MTT (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma) was added, followed by incubating for 3 h at 37°C. The solution was then discarded, and cells were suspended in 100 µL of DMSO (Dimethyl Sulfoxide, Junsei, Japan). Absorbance was calculated from optical density (OD_{540}) values measured using a microplate reader (BioTek Instruments, Korea). The assay was repeated three or more times and all data are the average ± STDEV.

Ames mutagenicity assay

The Ames mutagenicity assays were performed, according to the method of manufacturer recommendation (Xenometrix, Switzerland) (Flückiger-Isler and Kamber, 2012). Both tester strains, *Salmonella Typhimurium* TA98 and *Escherichia coli* WP2 *uvrA*, were grown in growth medium overnight at 37°C. The test cultures were exposed to the indicated concentrations of extracts for 90 min in liquid

Table 1. Zone of inhibition by plant extracts against bacterial strains.

Strains	Materials	Clear zone (mm)
S. aureus (CCARM3505)	Ampicillin	26.0 ± 0
	A. koreana	15.5 ± 1.1
	A. euchroma	15.9 ± 2.2

Source: Authors

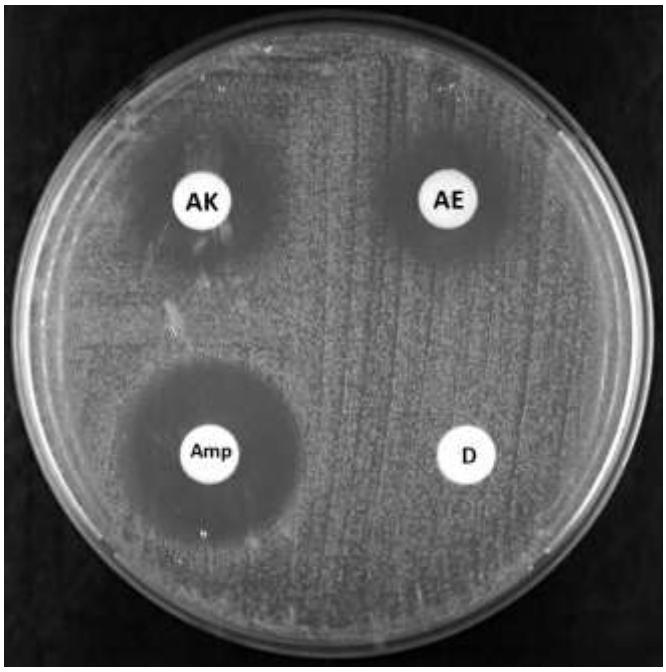

Figure 1. Antibacterial activity of *A. koreana* (AK) and *A. euchroma* (AE) extract against *S. aureus* (CCARM3505) with disk diffusion method. Bacteria with a McFarland standard of 0.5 were swabbed evenly onto plates. Disk containing 30 µL of ampicillin and extracts were placed onto the plates. D = DMSO; Amp = Ampicillin.
Source: Authors

minimal exposure media in a 24-well plate. After each well of the 24-well plates was added with 2.6 ml of indicator medium, 100 µL aliquots of culture were then dispensed into a 96-well plate. The 96-well plates were incubated at 37°C for 48 h. The number of positive (yellow) wells out of 48 wells per replicate was compared to the number of revertants from the negative control.

RESULTS AND DISCUSSION

Antibacterial test

The extracts of AK and AE showed clear zone sizes of 15.5 ± 1.1 and 15.9 ± 2.2 mm against *S. aureus*, respectively (Table 1 and Figure 1). Lactic acid bacteria (LAB), particularly those belonging to beneficial and non-pathogenic bacteria have traditionally been used in the food industry. Recent studies have shown their preventive effect against infection. For example, the incidence of infections and acute diarrhoea in children is reduced (Gleeson et al., 2011; Sur et al., 2011). In addition, LAB is widely used as functional foods, and LAB fermentation products and supernatant are also useful as cosmetic ingredients.

Streptococcus spp., *Lactobacillus* spp., and *Lactococcus* spp. are mainly applied for fermentation. Various substrates such as soybeans, fruit, and plants are used for culture (Izawa and Sone, 2014). With three *Lactobacillus* spp., AK and AE extracts were separately fermented for 72 h. Fermentation with AF showed a larger clear zone size of 19.2 ± 2.3 mm compared with non-fermented AK extract. In the case of AE extract, fermentation with AF also showed a prominent clear zone

Table 2. Zone of inhibition of 72 hours-fermented plant extracts against *S. aureus* (CCARM3505).

Extracts	Fermentation strains	Clear zone (mm)
		S. aureus
A. koreana	-	15.0 ± 0.9
	L. acidophilus (AD)	18.7 ± 3.2
	L. acidifarinae (AF)	19.2 ± 2.3
	L. acidipiscis (AP)	12.0 ± 0
A. euchroma	-	14.8 ± 1.1
	L. acidophilus (AD)	15.0 ± 2.1
	L. acidifarinae (AF)	16.6 ± 1.5
	L. acidipiscis (AP)	12.0 ± 0

Source: Authors

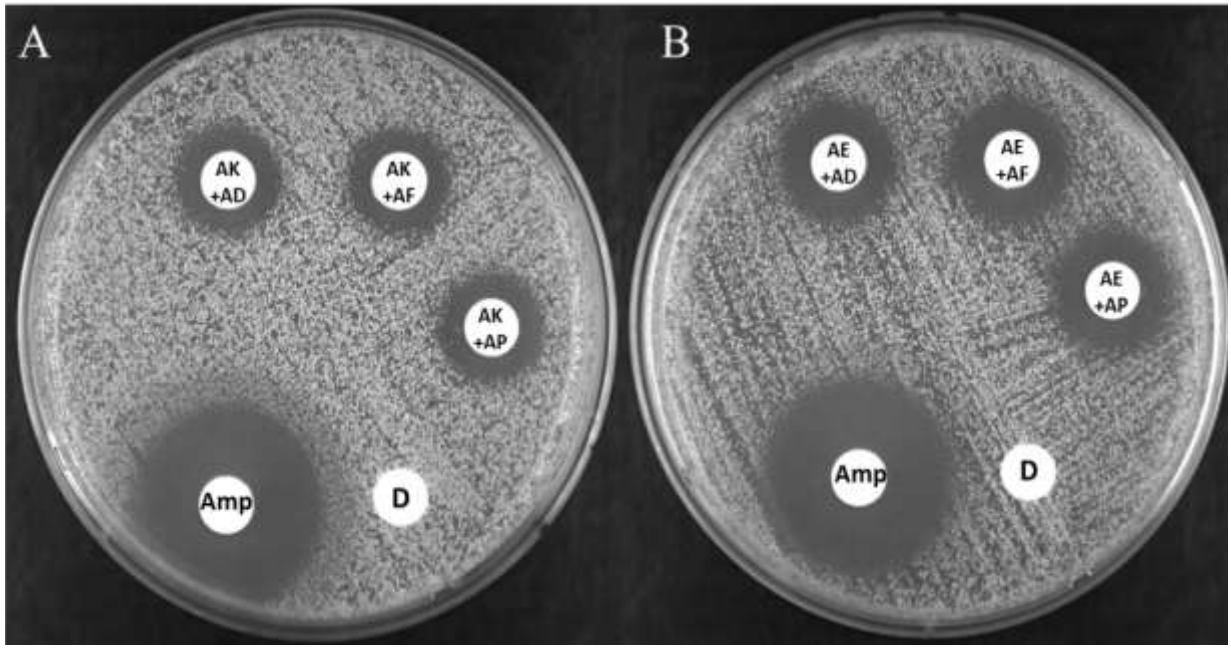

Figure 2. The antibacterial activities of 72 hours-fermented extract with *Lactobacillus* spp. against *S. aureus* (CCARM3505). (A) The extract of *A. koreana* (AK) fermented by *L. acidophilus* (AD), *L. acidifarinae* (AF), or *L. acidipiscis* (AP) for 72 h; (B) The extract of *A. euchroma* (AE) fermented by *L. acidophilus* (AD), *L. acidifarinae* (AF) or *L. acidipiscis* (AP) for 72 hours. D = DMSO; Amp = Ampicillin.
Source: Authors

size of 16.6 ± 1.5 mm (Table 2 and Figure 2). To test how long time of fermentation is most effective, AK and AE extracts were fermented with AF for indicated times. Both extracts showed clear zone sizes of 17.8 ± 2.6 and 16.4 ± 1.9 mm, respectively, when fermented for 72 hours (Table 3 and Figure 3).

Various combinations of the two fermented extracts were tested for synergistic effect. The largest clear zone size was expressed as 16.4 ± 2.8 mm by AK+AF: AE+AF=3:2 combinations (Table 4 and Figure 4). AK and AE extracts showed antibacterial activity against *S. aureus* but to make the extract showed higher activity, the fermentation by LAB was applied.

Fermentation with LAB is used in various fields, and in this study, three types of LAB were tested. Among the three LAB, the largest clear zone was observed in the fermented extract with AF, and the greatest clear zone was observed with 72 h fermentation, which was selected

Table 3. Zone of inhibition by fermented extracts for indicated time against *S. aureus* (CCARM3505).

Strains	Materials	Clear zone (mm) Fermentation time (h)			
		24	48	72	96
S. aureus (CCARM3505)	AK+AF*	15.6 ± 1.9	15.3 ± 1.8	17.8 ± 2.6	15.2 ± 0.3
	AE+AF*	15.6 ± 2.1	15.4 ± 2.5	16.4 ± 1.9	14.5 ± 0

Source: Authors

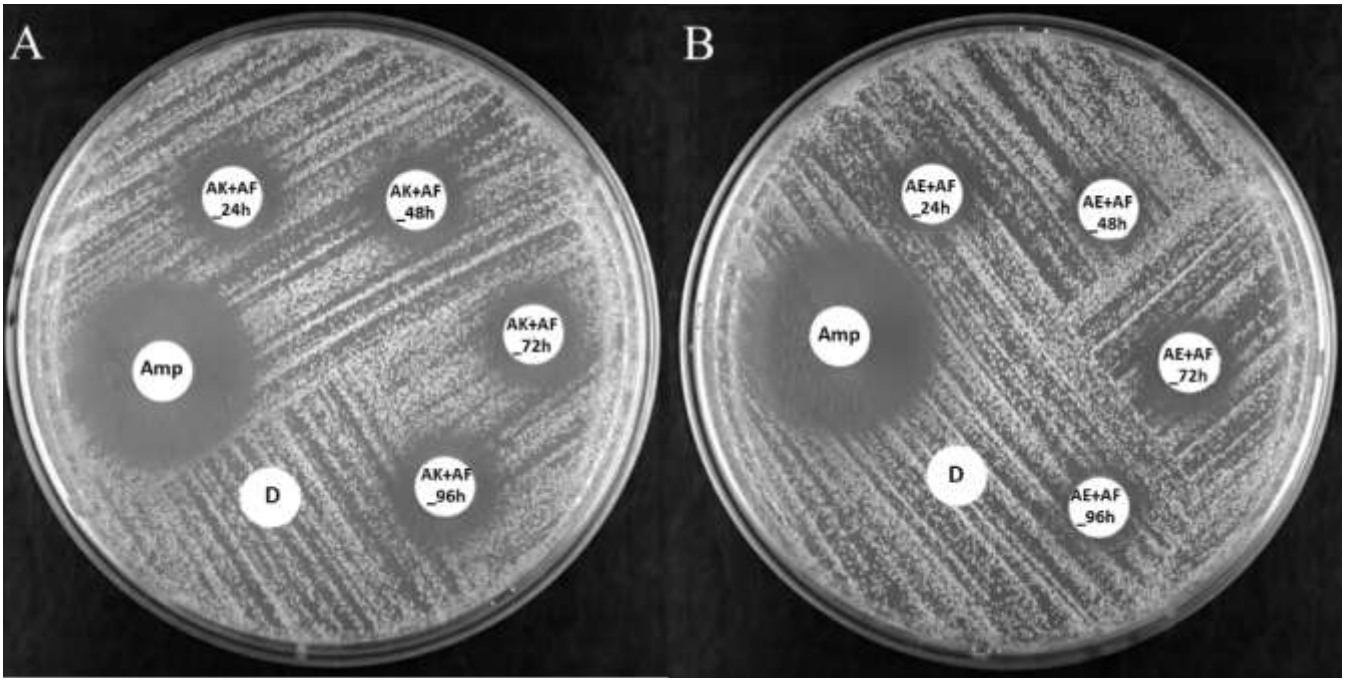

Figure 3. The antibacterial activities of fermented extract with *L. acidifarinae* against *S. aureus* (CCARM3505). (A) The antibacterial activities of AK fermented for indicated time (24, 48, 72 and 96 h); (B) The antibacterial activities of AE fermented for indicated time (24, 48, 72 and 96 h). D = DMSO; Amp = Ampicillin.
Source: Authors

Table 4. Zone of inhibition by combined treatment of fermented extracts against *S. aureus* (CCARM3505).

Ratio of *AK+AF:*AE+AF	Clear zone (mm) *S. aureus*
5:0	14.9 ± 2.4
4:1	16.1 ± 1.9
3:2	16.4 ± 2.8
2:3	14.2 ± 3.1
1:4	14.5 ± 1.8
0:5	15.5 ± 2.0

*AK+AF: *L. acidifarinae* (AF) fermented *A. koreana* extract, AE+AF: *L. acidifarinae* (AF) fermented *A. euchroma* extract.
Source: Authors

as the best fermentation condition. AF was first identified in 2005 (Vancanneyt et al., 2005), but research about AF has not been largely conducted. Therefore, the increased antibacterial activity by fermentation with AF found in this study appeared to be meaningful.

Biofilm formation inhibition

Surface adhesion of bacteria is an essential step and is necessary for bacteria to infect host in their environment. The role of biofilm is to attach to abiotic surfaces, epitheliums, and interfaces in multicellular organisms (Berne et al., 2015).

Biofilm serves to promote bacteria survival by preventing antibiotic activity and host immune responses,

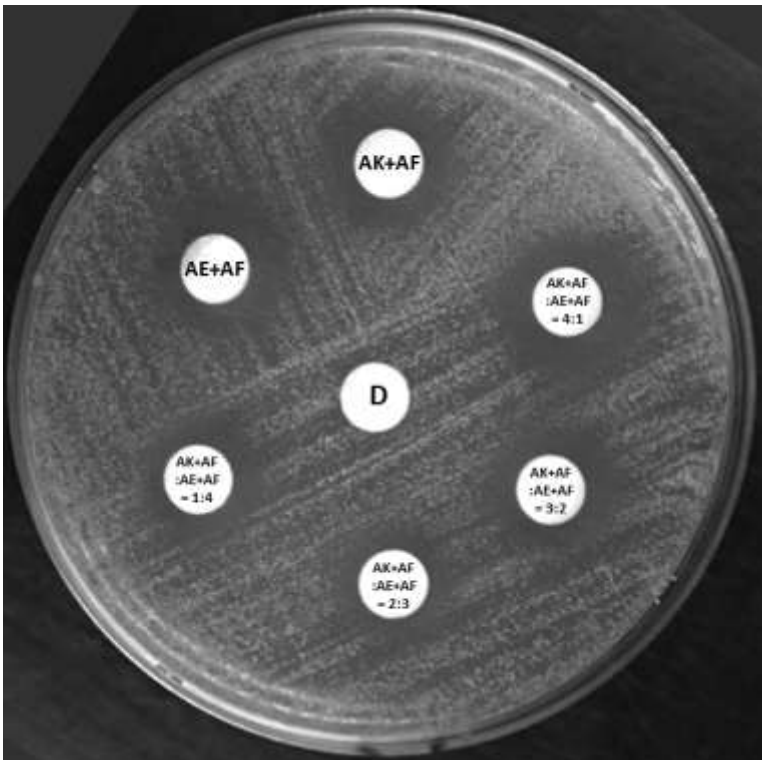

Figure 4. The antibacterial activity of combined treatment of fermented extracts against *S. aureus* (CCARM3505), D = DMSO; Amp = Ampicillin. Source: Authors

so it is considered as a good target for the biofilm forming pathogen (Schulze et al., 2021). To examine the potential anti-biofilm formation effect of the fermented extracts, *S. aureus* was cultured in the presence of the fermented extracts and biofilm formation was detected using crystal violet biofilm assay. Each fermented extract and combination of both fermented extracts dramatically decreased *S. aureus* biofilm formation by over 50%. The most prominent anti-biofilm formation activity was observed with AE extract fermented by AF. 100 µg/mL of AK and AE extracts fermented with AF inhibited biofilm formation by 44.19 ± 13.06 and 18.57 ± 4.81 compared with untreated bacteria, respectively. Combined treatment of both fermented extracts showed 35.55 ± 6.53% inhibition of *S. aureus* biofilm formation (Figure 5). These results suggest that AF-fermented AK and AE extracts are good candidates to control the biofilm forming pathogenic *S. aureus* (Ames et al., 1973).

Cell viability

Although AK extract, AE extract, and fermented extracts exhibit antibacterial effects against *S. aureus*, it is important to test toxicity with normal mammalian cell lines including HaCaT (Human keratinocyte cell line). AK extract showed cell viability lower than 20% at 250 µg/mL treatment (Figure 6A) but AK extract fermented with AF for 72 h showed reduced toxicity with higher than 80 and 60% of cell viability at 250 and 1000 µg/mL treatment, respectively (Figure 6C). AE extract and fermented AE extract did not influence the cell's viability (Figure 6B, 6D).

Ames test

The Ames test was repeated three times without S9 metabolic activation. Fermented extract and combined treatment did not show any mutagenic activity in TA98 and WP2 *uvrA* strains without metabolic activation (Table 5). None of the investigated fermentations showed any potential mutagenic effects.

Conclusion

This manuscript showed that *A. koreana* and *A. euchroma* extracts exhibited the antibacterial effect against *S. aureus*. Fermented extracts by *L. acidifarinae* showed improved antibacterial effect and anti-biofilm formation activity against *S. aureus* with reduced animal

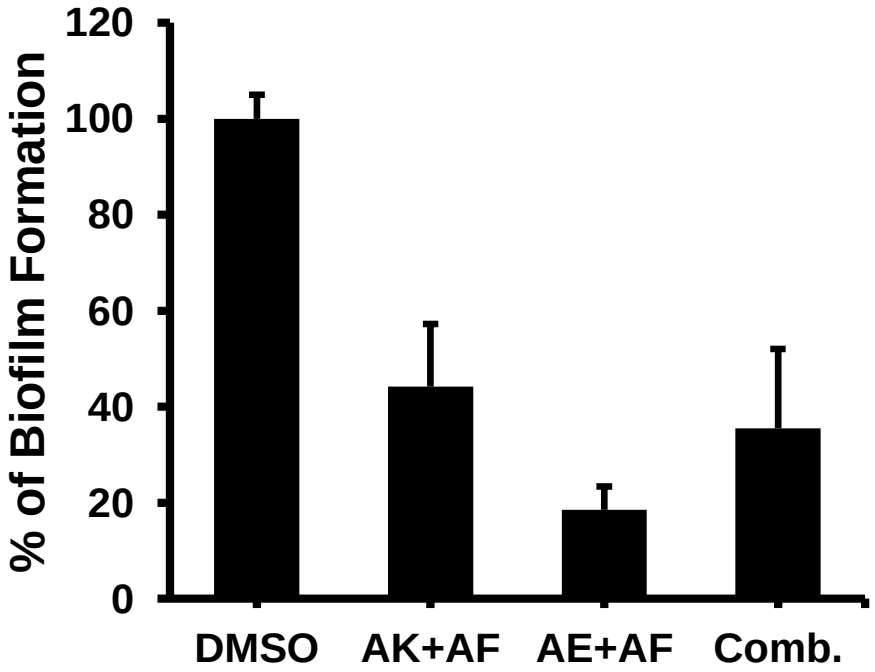

Figure 5. Fermented extract inhibited *S. aureus* biofilm formation. Effect of fermented extracts (100 µg/mL) against *S. aureus* biofilm formation was detected using crystal violet solution. AK+AF (72 h-fermented), AE+AF (72 h-fermented) and combined treatment of both fermented extracts (Comb., AK+AF: AE+AF = 3:2) were used.
Source: Authors

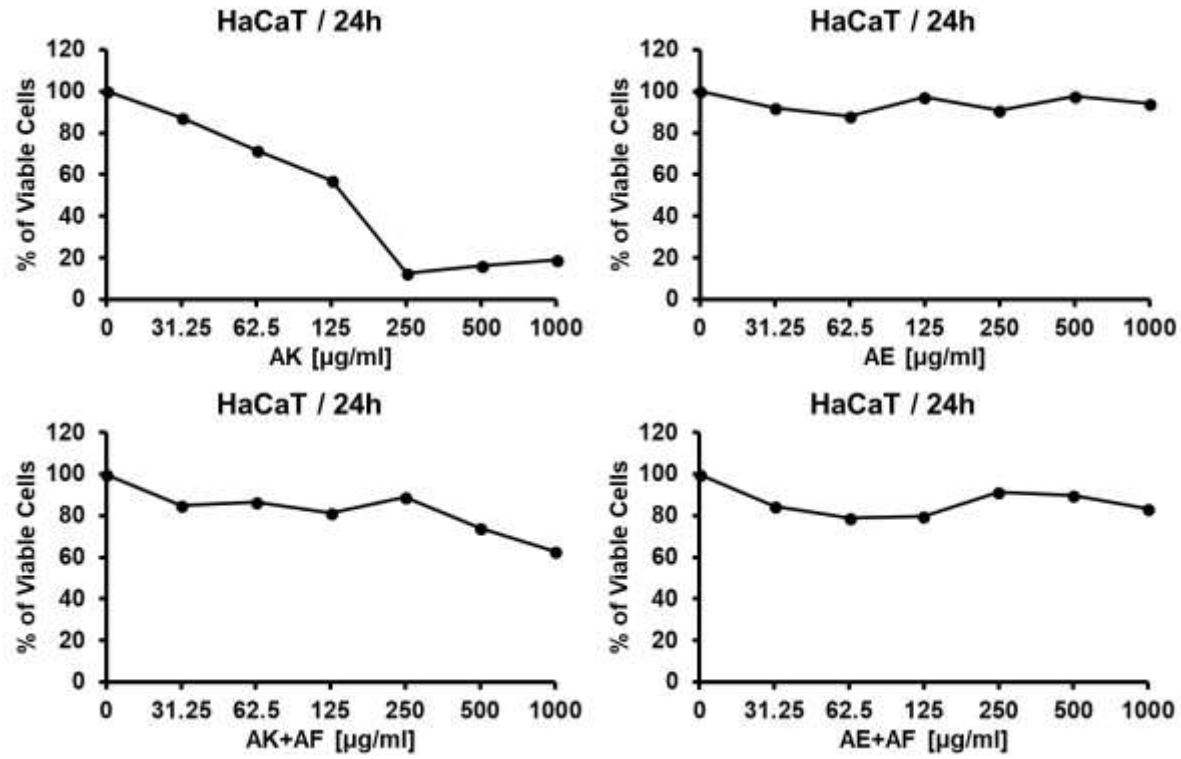

Figure 6. Fermented extracts reduced the toxicity of AK extracts. Effects of (A) AK, (B) AE, (C) AK+AF (72 h-fermented) and (D) AE+AF (72 h-fermented) on the growth of HaCaT cells were tested.
Source: Authors

Table 5. Mutagenicity of fermented materials toward *S. typhimurium* TA98 and *E. coli* WP2 *uvrA* strains without metabolic activation.

Materials	Dose (µg/mL)	Number of revertants (Mean)	
		***S. typhimurium* TA98**	***E. coli* WP2 *uvrA***
DMSO	-	4	8
*AK+AF	40	8	4
*AE+AF	40	36	4
AK+AF:AE+AF =3:2	80	16	8

*AK+AF: *L. acidifarinae* (AF) fermented *A. koreana* extract, AE+AF: *L. acidifarinae* (AF) fermented *A. euchroma* extract.
Source: Authors

cell toxicity compared with non-fermented extract.

Funding

This research was funded by the TIPA, Korea Technology and Information Promotion Agency for SMEs (#1425153495).

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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