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INVESTIGATION OF BIOLOGICAL EFFECTS OF EDIBLE FILMS DEVELOPED WITH *Citrus medica* L. var. *sarcodactylis* ETHANOL EXTRACT AND LACTIC ACID BACTERIA LYSATE AND POTENTIAL USE IN FOOD AND AQUACULTURE

SUMMARY

Edible films (EF) have gained significant attention as a sustainable alternative to synthetic packaging, offering potential benefits in food preservation, environmental impact reduction, and increased recyclability. The aim of the study was to investigate the antimicrobial activity of EF containing *Citrus medica* L. var. *sarcodactylis* (fingered citron) ethanol (CE) extract and lactic acid bacteria (LAB) lysate. In this context, the inhibitory effect of CE extract on some food-borne and fish-borne microbial strains was evaluated by disc diffusion and microdilution assay. Additionally, the biological activities of EF prepared using natural components (CE extract and LAB lysate) were determined by well diffusion test. Disc diffusion assay results indicated that the highest inhibition zone diameter was recorded as 10.63 mm against *Escherichia coli* O157:H7, while the lowest value was determined as 7.91 mm against *Yersinia ruckeri*. The lowest value for minimum inhibitory (MIC) and bactericidal concentration (MBC) was determined against *Streptococcus agalactiae* Pas. Ins. 55118 (5 mg/ml), and the highest value was determined on *Vibrio anguillarum* M1 (40 mg/ml). In addition, the antimicrobial activity tests of the developed EF formulation stated that the highest inhibition zone diameter was measured as 11.37 mm against *E. coli* O157:H7, while the lowest value was determined as 4.49 mm against *V. anguillarum* M1. As a result, it was revealed that EF developed with CE extract and LAB lysate with good antimicrobial activity may have potential use as natural preservatives in the food and aquaculture industries.

Keywords: Fingered citron, Probiotic, Bioactive component, Pathogen, Natural preservative

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INTRODUCTION

Edible films (EF) are thin sheets that are applied to foods as a protective layer and can also be consumed (Galus *et al*., 2020). EF has attracted great attention with their potential to be used as an alternative to synthetic films. This can make a significant contribution to reducing environmental pollution. It can also simplify the structure of packaging materials by acting as a barrier. EF can increase recyclability compared to traditional environmentally unfriendly packaging (Bourtoom, 2008). Nowadays, solutions have been developed to prevent food spoilage and increase the nutritional value of food products by adding bioactive compounds and microorganisms (e.g. probiotics) to coating materials. These applications stand out as important techniques affecting inhibition of pathogens, product quality, ripening, thickening effect and shelf life (Díaz-Montes and Castro-Muñoz, 2021).

Plants are a rich source of antimicrobial substances that are effective against many bacteria. Some of the important components found in plants, polyphenols, are the most potent antimicrobial compounds. These components tend to have synergistic or antagonistic effects on pathogens (Hochma *et al*., 2021). Plant-based antimicrobials are found in plant parts such as roots, stems, leaves, flowers, fruits and seeds, providing a powerful alternative to antibiotics (Li *et al*., 2024). In this context, *C. medica* L. var. *sarcodactylis*, which attracts attention with its appearance, is a member of the Rutaceae family. It is mostly spread in Taiwan, China, Malaysia, India, Vietnam. In recent years, its cultivation has also begun in Türkiye. Its bark, leaves and root have been used as a folk medicine in the treatment of edema, hepatitis, headache, stomachache, arthritis and rheumatism (Chan *et al*., 2010). *C. medica* L. var. *sarcodactylis* leaves have been reported to have anti-inflammatory, antifungal, antioxidant and antimicrobial activities (Chan *et al*., 2010; El Hawary *et al*., 2022; Wu *et al*., 2021). However, most of the by-products of the plant, such as flowers and leaves, are discarded, leading to waste of valuable bioactive resources. It has been reported that Limonene is high among the main components of *C. medica* L. var. *sarcodactylis* leaves (Wu *et al*., 2021). It has been stated that the basic mechanism of antibacterial activity of limonene is to change the permeability of the membrane by causing damage to the cell membrane. Additionally, studies have shown that limonene not only inhibits pathogenic microbes that cause disease, but also protects various food products from potential contaminants (Gupta *et al*., 2021).

Probiotics, especially lactic acid bacteria (LAB), have an important place in the food industry (Abedin *et al*., 2024). LAB can be used to control microorganisms to prevent food spoilage. Some of these LAB strains, primarily from the Lactobacillus and Bifidobacterium genera, can promote health, known as probiotics (Mokoena *et al*., 2021). Also, the advantages provided by LAB in preventing or controlling infectious diseases in cultured fish are also taken into consideration. LAB are important members of the fish microbiota and have an antagonistic effect against opportunistic pathogens, fungi and viruses that pollute the aquatic ecosystem and cause diseases in aquatic organisms (Chizhayeva *et al*., 2022). For these reasons, LAB is therapeutically useful against many foodborne illnesses and is a highly recommended alternative for extending product shelf life (Zapaśnik *et al*., 2022). *Limosilactobacillus fermentum* strains produce various potent antimicrobial components that can be applied as food preservatives or alternatives to antibiotics (Naghmouchi *et al*., 2020). Therefore, it has been reported that edible coatings containing *L. fermentum* can increase the health value while increasing the shelf life of food (Najabi *et al*., 2024). In this study, the effects of EF developed using CE extract and LAB lysate on food quality and safety were investigated. In the first stage, the antibacterial activity of CE extract on some food-borne and fish originated pathogens was evaluated. Then, the antimicrobial effects of EF obtained with carrageenan and natural components (CE extract and LAB lysate) were investigated and its potential for use as a natural preservative for food and aquatic products was determined.

MATERIAL AND METHODS

Sample Collection and Extraction Method with Ethanol Solvent

Fresh *C. medica* L. var. *sarcodactylis* leaves were collected from the Muğla Köyceğiz Plant World located in Türkiye, in October 2022. The leaves were then air-dried in a dark environment. After drying, the leaf samples were ground into a fine powder. Extraction was carried out using ethanol as the solvent. A homogeneous mixture, consisting of 10 grams of leaf powder and 30 milliliters of water, was prepared and subjected to extraction in a water bath at 70°C for 24 hours. Following extraction, the remaining solvent was evaporated to yield the final extract. The prepared CE extract was stored at 4°C (Figure 1.).

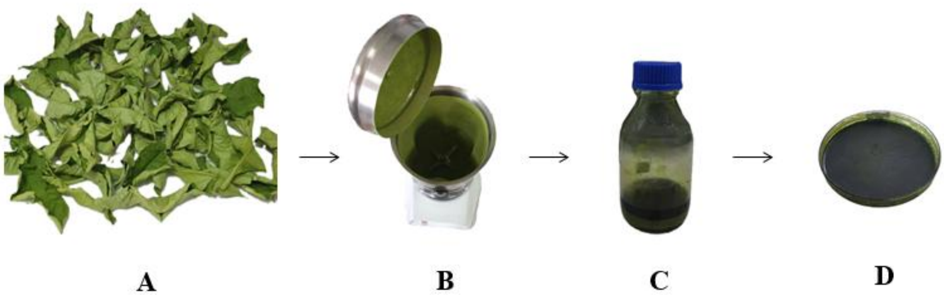

Figure 1. Extraction steps of *Citrus medica* L. var. *sarcodactylis* leaves: Dried leaf samples (A), grinding process (B), ethanol extraction (C), and concentrated extract (D).

Culture Conditions of Test Microorganisms

The biological activity of the CE extract on some pathogenic bacteria was determined. *Escherichia coli* O157:H7 (food-borne) and *Aeromonas hydrophila* ATCC 19570 (fish pathogen) were grown in Nutrient Broth at 37˚C. *Streptococcus agalactiae* Pas. Ins. 55118 (fish pathogen), *Yersinia ruckeri* (fish pathogen) were incubated in Tryptic Soy Broth at 37˚C. Fish pathogens *Vibrio anguillarum* A4 and *V. anguillarum* M1 were cultured in Tryptic Soy Broth-NaCl

(2%) medium at 25˚C. All bacterial cultures were maintained for 24 h incubation under appropriate conditions to ensure optimum growth.

Antibacterial Assay

The antimicrobial properties of the CE extract were evaluated using the disc diffusion assay (Tasbası and Asan-Ozusaglam, 2024). A microbial suspension standardized to a density of 0.5 McFarland was spread onto the solid medium. Sterilized 6 mm discs were placed in the medium three times and the extract solution (20 µl) prepared at a concentration of 100 mg/ml (2 µg/disc) was applied to each disc. The plates were then incubated for 24 hours under the conditions stated above. Positive control antibiotic disc contained Kanamycin (30 µg). Negative control used was DMSO.

Micro-Dilution Method

The micro-dilution assay was used to determine the minimum inhibition and bactericidal concentrations (MIC and MBC) of the CE extract on the above-mentioned bacteria (Tasbası and Asan-Ozusaglam, 2024). Firstly, solution (medium, bacterial suspension and CE extract) was prepared in tubes for the MIC value. The tubes were then incubated for 24 hours at the appropriate temperature specified above. As a result of incubation, the concentration in the tube with no microbial growth was determined as MIC. For the MBC value, 5 µl samples were taken from tubes containing various concentration of the extract (2.5, 5.0, 10.0, 20.0, 40.0, 80.0 mg/ml) and applied to solid media. Then, microbial growth was observed by incubating 24 hours. At the end of this period, the concentration at which microbial growth stopped completely was defined as MBC value.

Determination of Antibacterial Activity of Edible Film Containing Extract and Lactic Acid Bacteria

EF developed with CE extract and LAB components was tested against the bacteria by modifying the procedure described by Venkatachalam *et al*. (2023). In the formulation where EF were prepared, CE extract (10% w/v), LAB lysate isolated from human milk (*L. fermentum* MA-7), carrageenan gum and glycerol (1% and 3% w/v) were used. The present study includes four experimental groups: Gum (G/Control), Gum-Lysate (GL), Gum- CE Extract (GCE), and Gum- CE Extract-Lysate (GCEL). In the determined groups, the final volume of the groups without LAB was completed with water, while the final volume of the other groups was completed with LAB lysate. The film mixtures were mixed with a magnetic stirrer at 90˚C for 30 minutes. The biological activity of the homogenized EF was first determined. Then, drying was performed in a Pasteur oven until constant weight was reached for the analysis of mechanical properties. For the biological activity of the film mixtures, sterilization was performed with a filter (0.45 µm). The culture adjusted to 0.5 McFarland (100 µl) was added to each petri dish and the film mixture (100 µl) was added to the wells with a diameter of 6 mm. The petri dishes were incubated and kept in the environment and temperature conditions suitable for pathogens. Then, the diameters of the inhibition areas were determined using calipers.

Statistical analysis

One-way ANOVA test was performed with the SPSS program (GNU) to determine significant differences between the values of antimicrobial activity. The statistical significance of the change between the determined mean values was assessed using the Tukey test ($p< 0.05$).

RESULTS AND DISCUSSION

Pathogenic microorganisms have been a threat since the beginning of human history and have been one of the leading causes of disease and death in humans (Górniak *et al*., 2019). In this context, the antimicrobial activity of CE extract containing biologically active components against pathogens was evaluated. The results obtained against various microbial strains important for the safety of the food and aquaculture industries are given in Table 1.

Table 1. Antibacterial activity and micro-dilution assay of *C. medica* L. var. *sarcodactylis* leaf extract

Microorganisms	Inhibition Zone Diameters (SD±mm)		MIC (mg/ml)	MBC (mg/ml)
	CE Extract	**Kanamycin (30 µg)**		
E. coli O157:H7	10.63 ± 0.39^{a}	15.85 ± 0.58^{a}	10	20
S. agalactiae Pas. Ins. 55118	$9.69\pm0.51^{a,b}$	19.21 ± 3.06^{a}	5	5
V. anguillarum A4	8.85 ± 0.55^{b}	6.28 ± 0.17^{b}	20	20
V. anguillarum M1	10.11 ± 0.21^{a}	$-^{c}$	40	40
A. hydrophila 19570 ATCC	$8.33\pm0.41^{c,b}$	10.58 ± 0.24^{d}	5	20
Y. ruckeri	$7.91\pm0.20^{c,b}$	$12.50\pm1.43^{a,d,e}$	10	40

*CE: *C. medica* L. var. *sarcodactylis* ethanol, MIC: Minimal inhibition, MBC: Minimal bactericidal concentrations, CE Extract F(*Sig*): 20.962(0.000), Kanamycin F(*Sig*): 71.541(0.000).

According to the Table 1, *E. coli* O157:H7 (10.63 mm) and *V. anguillarum* M1 (10.11 mm) stand out among microbial strains with similar and high inhibition zone diameters. No statistically significant difference was found between these food-borne and fish pathogens ($p>0.05$). However, a significant difference was determined when compared with other microbial strains ($p<0.05$). The lowest inhibition zone diameter was determined as 7.91 mm against *Y. ruckeri*. It was obtained that CE extract was more effective against *V. anguillarum* A4 than the traditional antibiotic Kanamycin (30 µg, positive control). In fact, although *V. anguillarum* M1 was resistant to Kanamycin, the CE extract showed antimicrobial activity on this bacterium with an inhibition zone diameter of 10.11 mm, which was an important result. On the other hand, although the inhibition zone diameters of the CE extract are low compared to Kanamycin, the effect can be increased with appropriate concentration adjustments. CE extract, which is not a pure compound, shows good potential for antimicrobial activity with good inhibition zone diameters. It was determined that DMSO solvent (negative control) had no effect on the tested microorganisms.

One of the most dangerous foodborne pathogens, *E. coli* O157:H7, poses a major threat to human health, public safety, and economic property worldwide (Bai *et al*., 2022). *V. anguillarum* is a pathogenic bacterium that poses a significant risk to the seafood industry, can lead to foodborne diseases, and can cause infections known as "vibriosis". Vibriosis causes serious problems both from a food safety and economic point of view, and prevention and treatment methods are usually quite costly (Hickey and Lee, 2018). Although the highest two inhibition zones were obtained against these pathogenic bacteria, the lowest MIC and MBC values of the CE extract was identified on *S. agalactiae* Pas. Ins. 55118. According to the MIC and MBC values, CE extract exhibited the highest antimicrobial activity on *S. agalactiae*, as compared to the other bacteria that were tested. These findings indicate that CE extract can be used as an effective antimicrobial substance especially against certain food-borne and fish pathogens.

The studies conducted with *C. medica* L. var. *sarcodactylis* leaves are limited. In addition, it has been reported that the inhibition zone diameters of dry and wet lemon grass ethanol extracts against *E. coli* at concentrations of 250, 500 and 1000 ppm varied between 3.10-5.40 mm (Nyamath and Karthikeyan, 2018). The MIC values of *C. hystrix* leaf and *C. maxima* methanol red leaf extracts against *E. coli* MTCC 40 were determined as 50 and 75 mg/ml. The antimicrobial activity of the leaf extract was determined to be directly related to the bioactive compounds they contain (Abirami *et al*., 2013). In this study, it was determined that CE extract with lower MIC and MBC values (10 and 20 mg/ml) had stronger antimicrobial activity on *E. coli* O157:H7. In a study conducted by Asan-Ozusaglam *et al*. (2021), the inhibition zone diameters of methanol extract prepared from kumquat leaves against *V. anguillarum* A4 and M1 were reported as 24.41 and 24.80 mm. MIC and MBC values on microbial strains were also determined as 25 mg/ml. Comparing this study with our current study, it was determined that CE extract had lower inhibition zone diameter against *Vibrio anguillarum* M1 and A4 strains, but showed higher antimicrobial activity on *Vibrio anguillarum* A4 with lower MIC and MBC values (20 mg/ml).

The antibacterial activity of EF formulations was determined using the well diffusion method and the results are given in Table 2. Gum (G/control) group did not show inhibitory activity against the tested microorganisms. In the Gum-Lysate (GL) group, inhibition zone diameters were determined against other test microorganisms except *Y. ruckeri*. The highest inhibition zone diameter was determined as 10.11 mm against *A. hydrophila* 19570 ATCC. In the Gum-Extract (GE) group, 1.63 mm of inhibition zone diameter was detected against *E. coli* O157:H7, while no activity was observed against other test microorganisms. Statistical analysis showed significant differences between all tested EF groups for *E. coli* O157:H7 ($p<0.05$). Also, a significant difference was found between the GL and GCEL groups ($p<0.05$). In addition, it was observed that the inhibition zone diameters increased significantly between the GL and the GCEL groups. In GCEL group, the inhibition zone diameters of CE extract on the tested bacteria except for *Y. ruckeri* were higher than GL group. While there was no inhibitory effect against *Y. ruckeri* in G, GL and GCE groups, antimicrobial activity was observed in GCEL group with an inhibition zone diameter of 6.09 mm. When evaluated for all tested bacteria, antibacterial activity or higher antimicrobial activity was detected in the GCEL group compared to the other EF groups. This may be due to the synergetic effect of *L. fermentum* MA-7 lysate and CE extract. These results indicate that CE extract and LAB lysate may provide a natural antimicrobial solution against food and fish pathogens with their rich bioactive compound contents. In particular, the combination of CE extract and LAB lysate is promising for functional applications in food, pharmaceutical and aquaculture industries.

Table 2. Antibacterial activity of EF groups prepared with CE extract and/or LAB lysate

Microorganisms	**Inhibition Zone Diameters (SD±mm)**				
	Gum (G/Control)	**Gum-Lysate (GL)**	**Gum-CE Extract (GCE)**	**Gum-CE Extract-Lysate (GCEL)**	**F (*Sig*)**
E. coli O157:H7	-[a]	3.27±0.10[b]	1.63±0.30[c]	11.37±0.36[d]	1328.951 (0.000)
S. agalactiae Pas. Ins. 55118	-[a]	2.36±0.12[b]	-[a]	5.59±0.49[c]	328.084 (0.000)
V. anguillarum A4	-[a]	2.73±0.11[b]	-[a]	4.49±0.16[c]	1570.882 (0.000)
V. anguillarum M1	-[a]	4.24±0.33[b]	-[a]	6.30±0.55[c]	294.746 (0.000)
A. hydrophila 19570 ATCC	-[a]	10.11± 0.14[b]	-[a]	10.25±0.18[b]	7701.243 (0.000)
Y. ruckeri	-[a]	-[a]	-[a]	6.09±0.07[b]	22578.270 (0.000)

* CE: *C. medica* L. var. *sarcodactylis* ethanol, -: no inhibition zone diameter

Febriana *et al*. (2021) reported that edible coatings protect fillets from spoilage by reducing water vapor, aroma, and lipid transmission. In their study, they examined the effects of adding *C. hystrix* leaf essential oil to the gelatin coating on the shelf life of red sea bream fish, which deteriorates rapidly due to its high protein and water content. According to the research results, the edible coating method was determined to be an effective approach in extending the shelf life of red sea bream fillets at room temperature and preventing bacterial growth. In addition, Azizah *et al*. (2023) evaluated the effects of adding pectin and lemongrass essential oil to gelatin film on its properties, chicken breast quality preservation potential and antimicrobial activity. It was revealed that the edible film had an inhibitory effect on *Salmonella* and its application to chicken breast could maintain its quality during storage. In another study, antimicrobial film was obtained from *C. aurantifolia* leaf extract. The addition of leaf extract to the film was found to have effective activity in growth inhibition against *Staphylococcus aureus* (Asria and Elizarni, 2015). In our study, it was determined that edible film obtained from CE extract and/or LAB lysate could show significant inhibitory activity against pathogens that can be observed especially in the food and aquaculture industries.

CONCLUSIONS

The use of CE extract as a ‘natural’ antibacterial agent in the food and aquaculture industry and the potential application areas of the prepared EF were investigated. Consumer interest in natural preservatives that can replace chemical additives has increased significantly in recent years. Our study revealed that EF containing bioactive components (CE extract and LAB lysate) exhibited a strong antimicrobial effect against important pathogens in the food and aquaculture industry. This offers a solution for the development of films with natural and antimicrobial properties that can prevent spoilage by extending the shelf life of foods. The statistical significance of the CE extract's antimicrobial activities underscores its potential as a promising natural additive for food and aquaculture applications.

This effect may be due to limonene, a terpene found in high amounts in FC leaves. In addition, it has been determined that CE extract can lead to the development of new plant-based products as a safe option. The findings obtained from in-vitro studies of CE extract support the applicability of this extract in different areas such as food and aquaculture and show that it can form the basis for in-vivo research with cell culture. In this way, CE extract with antimicrobial activity offers potential application areas in various industries.

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