

AN INNOVATIVE APPROACH TO VARIOUS INDUSTRIAL APPLICATIONS: THE FINGERED CITRON

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The fingered citron (*Citrus medica* L. var. *sarcodactylis*), which originates from India, has spread to different parts of the world over time. In opposition to the increasing resistance to traditional antimicrobial agents, the application of alternative treatments developed using herbs has recently gained interest. The aim of this study is to investigate alternative applications of the fingered citron in the cosmetics and pharmaceutical industries. For this purpose, the antimicrobial activity of fingered citron leaf water extract (FCLWE) against test microorganisms was evaluated using a disk diffusion assay. Minimum inhibitory concentration (MIC) and minimum cidal concentrations (bactericidal - MBC or fungicidal - MFC) of the extract were also determined. For FCLWE, among the pathogenic test microorganisms, *Listeria monocytogenes* strain ATCC 7644 and *Staphylococcus aureus* strain ATCC 25923 showed the smallest and the largest inhibition zone diameters, with values of 7.06 and 11.57 mm, respectively. MIC and MBC or MFC values of FCLWE varied from 10 to 40 µg/µl. In addition, the sun protection factors (SPF) of FCLWE and a mixture of FCLWE and commercial sun creams at different concentrations were determined. The SPF value of FCLWE was found to be 25. The mixture of FCLWE and commercial sun cream at an amount of 10 ml yielded an SPF value of 12.26. Next, the biological activity of the cream formulation obtained with commercial cream, FCLWE and *Limosilactobacillus fermentum* MA-7 (cream-leaf water extract-probiotic - CWP) was investigated against test microorganisms using the well diffusion method. CWP exhibited inhibitory activity against *L. monocytogenes* ATCC 7644 (2.56 mm), *Candida glabrata* RSKK 04019 (2.81 mm), *C. albicans* ATCC 10231 (4.54 mm) and *S. aureus* ATCC 25923 (5.00 mm). It was observed that the cream formulation of CWP increased the diameters of the inhibition zone by creating a synergistic effect against all pathogenic test microorganisms. FCLWE may potentially be used as an alternative natural additive to chemical preservatives in the cosmetics and pharmaceutical industries.

Keywords: antimicrobial activity, *citrus*, cream formulation, probiotic, sun protection factor

1. Introduction

Although antibiotics are among the most preferred options when combating bacterial infections [1], their inappropriate and widespread use causes microorganisms to develop resistance, posing a threat worldwide [2]. The increasing microbial resistance to synthetic antimicrobial agents poses a global challenge and the demand for alternative medicines targeting resistant strains is on the rise [3]-[4]. Medicinal and aromatic antimicrobial agents that may be effective against resistant strains are under investigation [5]. The medicinal properties of plants have been passed down from one society to the next for centuries [6]. Antimicrobial agents found in plants have an enormous therapeutic potential [7]. The medicinal properties of plants are often specific to certain genera of plants and arise from secondary metabolites present in their structure. Secondary metabolites found in plants consist of coumarins, alkaloids, terpenes, fatty acids, tannins, steroids and flavonoids [7]-[8]. Plants are

alternative natural resources that exhibit antimicrobial effects [9].

Fingered citron (FC) is an evergreen species of tree belonging to the Rutaceae family [10]. Originating from India, over time it has spread to different regions around the world [11]. This species possesses bioactive compounds such as flavonoids, terpenes, neolignans, coumarins and alkaloids [12]-[14]. Clinically, it has demonstrated various health effects, including hypoglycemic properties, efficacy in managing type 2 diabetes, hepatoprotective activity, relief from stomach pain, expectorant properties, potential anticancer activity and immunomodulatory effects [12]-[15].

The skin, which is crucial in protecting internal organs from external factors, is the largest sensory organ in our body. Being constantly in contact with external factors, it is exposed to possible dangers posed by harmful microorganisms. The first line of defense of the body against harmful microorganisms is the skin. The specific microbial flora on the skin protects against adverse environmental conditions [16]. Probiotics, on the

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other hand, contain live microorganisms that alter the skin flora of the host [17]. Probiotic microorganisms are used to reduce skin inflammation, regenerate the skin microbiota, delay aging and treat diseases such as dermatitis [18]-[19]. The fermentation broth called "lactic acid bacteria ferment" according to the International Nomenclature of Cosmetic Ingredients (INCI) is used in the production of various cosmetic ingredients [20]. For these reasons, both oral and topical probiotics are used to treat skin diseases and improve skin health by regulating microbial flora [16]-[21].

The sun serves as one of our primary energy sources, enabling life to thrive on Earth [22]. Working environments and outdoor areas are places where the risk of exposure to the sun's ultraviolet radiation is high [23]. When exposed to ultraviolet radiation from the sun, dermatitis, premature aging, cancer, tanning and sunburn can occur in the human body [22]-[24]. Ultraviolet light is divided into 3 regions, namely UVA (400-320 nm), UVB (320-290 nm) and UVC (290-200 nm) [25]. It is crucial to promote the use of photoprotective agents to mitigate the detrimental effects of ultraviolet radiation [26]. Certain components with photoprotective properties reduce the harmful effects of ultraviolet radiation on the skin. For this reason, the protective properties of plant extracts on the skin are being investigated. Bioactive molecules such as flavonoids and carotenoids contained in plant-based cosmetic products protect the skin from sunlight [27].

In this study, the antimicrobial activity of fingered citron leaf water extract (FCLWE) obtained using water as a solvent against clinical and foodborne pathogens as well as some probiotic candidate strains such as lactic acid bacteria (LAB) was tested. Sun protection factor (SPF) values of FCLWE and cream-FCLWE mixtures at different concentrations were measured to determine their potential application in the cosmetics industry as a natural alternative to chemical preservatives. In addition, the potential of new cream formulations prepared with FCLWE and the probiotic *L. fermentum* MA-7 in developing natural products by replacing chemical products in both the cosmetics and pharmaceutical industries was investigated.

2. Experimental

2.1. Plant material

FC leaf was obtained from Köyceğiz Plant World (Muğla-Türkiye) in October 2022 (Figure 1.A).

2.2. Preparation of leaf water extract

FC leaves (Figure 1.B) were dried in dry conditions in the absence of light. The ground leaves were extracted with water in a water bath (80 °C) over 24 hours (Figure 1.C). The FCLWE was filtered before being evaporated and concentrated to 100 mg/ml using dimethyl sulfoxide (DMSO) then refrigerated at +4 °C until used.

2.3. Preparation of bacterial strains

In this study, four pathogens and five LAB were used. All strains tested (*Staphylococcus aureus* ATCC 25923 in a nutrient broth, *Listeria monocytogenes* ATCC 7644 in Tryptic Soy Broth, *Limosilactobacillus fermentum* MA-7, *Limosilactobacillus fermentum* MA-8, *Lactobacillus gasseri* MA-1, *Lactobacillus delbrueckii* MA-9, *Limosilactobacillus vaginalis* MA-10 in De Man-Rogosa-Sharpe agar at 37 °C as well as *Candida glabrata* RSKK 04019 and *Candida albicans* ATCC 10231 in Yeast Peptone Dextrose at 30 °C) were incubated at the specified temperature and in a particular environment for 24 hours.

2.4. Determination of antimicrobial activity

The antimicrobial activity of the FCLWE was determined by the disk diffusion method [28]. The concentration (0.5 McFarland) of test microorganisms was prepared and the prepared suspension (100 µl) spread onto the agar medium. 3 replicates of sterile disks (6 mm) were placed on the agar medium. For the pathogens, 20 µl (2 mg/disk) of FCLWE was dripped onto each disk. For the LAB, 5 µl (0.5 mg/disk), 10 µl (1 mg/disk) and 20 µl (2 mg/disk) of FCLWE were dripped onto the disks. The Petri dishes were incubated at the specified temperature for 24 hours as mentioned above.



Figure 1: Samples of fingered citron leaves extracted with water

2.5. Minimum inhibitory and minimum bactericidal or fungicidal concentrations

The minimum inhibitory concentration (MIC) and minimum bactericidal (MBC) or fungicidal (MFC) concentration of the FCLWE were determined by the microdilution method [28]. The FCLWE and medium were added to the tubes and diluted before the test microorganism was added at a concentration of 0.5 McFarland. The samples extracted from MIC tubes were inoculated onto the solid medium. The FCLWE concentration at which the growth of microorganisms stopped was recorded as the MIC value. The concentration at which growth stopped in the solid medium incubated at the appropriate temperature for 24 hours was determined as the MBC or MFC value.

2.6. Determination of the SPF values of FCLWE and cream-FCLWE mixtures

The SPF values of the FCLWE and cream mixtures (cream-FCLWE) at different concentrations were determined using a Beckman Coulter spectrophotometer. The absorption values of FCLWE dissolved in ethanol (96%) were obtained in the range of 290 to 320 nm (every 5 nm). To determine the SPF values of the cream-FCLWE mixtures, 0.5 g of the extract and 1 g of the cream were added to a different tube before the volume was increased to 10 ml with distilled water. After diluting the mixture with ethanol (40%), sonication was performed before being filtered through Whatman Grade 1 filter paper. 3 replicates of each of the cream-FCLWE mixtures obtained at different amounts (2.5, 5.0, 10.0 ml) were measured in a spectrophotometer. SPF values were determined based on the formula below [29]:

$$SPF = CF \times \sum_{290}^{320} EE(\lambda) \times I(\lambda) \times Abs(\lambda) \quad (1)$$

where CF is a correction factor, $EE(\lambda)$ is the erythrogenic effect of radiation with wavelength λ , $I(\lambda)$ is the intensity of solar light at wavelength λ and $Abs(\lambda)$ is the spectrophotometric absorbance value at wavelength λ .

2.7. Antimicrobial activity of the cream formulation containing FCLWE and probiotic

The antimicrobial activity of the cream formulation enriched with FCLWE and probiotic (*L. fermentum* MA-7) was evaluated using a modified protocol by following the well diffusion method [30]. The cream formulation was prepared using a commercial cream, FCLWE and *L. fermentum* MA-7 [31]. Four different test sample groups were created for this experiment, namely one containing only the cream (control – C), another containing the cream and probiotic (CP), one more containing the cream and leaf water extract (CW) as well as a final group with the cream containing both the leaf water extract and probiotic (CWP).

2.8. Statistical analysis

The statistical analysis of the data concerning the biological activity was carried out using GNU SPSS software. The data points were confirmed by performing the One-Way analysis of variance (ANOVA) with Tukey's post-hoc test ($P < 0.05$ was considered to be statistically significant).

3. Results and discussion

The spread of pathogenic microorganisms and the development of resistance cause serious health problems [32]. The antimicrobial activity of the FCLWE against clinical, foodborne and yeast pathogens was determined using the disk diffusion assay and microdilution methods. The diameters of the inhibition zones obtained from the disk diffusion assay are given in Table 1. Data referring two antibiotics are also in Table 1. In case of FCLWE, the maximum diameters of the inhibition zones among the test microorganisms was 11.57 mm with regard to *S. aureus* ATCC 25923. The diameter of the inhibition zone appears to be good when *C. glabrata* RSKK 04019 (9.95 mm) and *C. albicans* ATCC 10231 (10.83 mm) were tested, which were used as fungal test microorganisms. *L. monocytogenes* ATCC 7644 exhibited the smallest diameter of the inhibition zone, that is, 7.06 mm ($P < 0.05$). When evaluated statistically, the difference between *S. aureus* ATCC 25923, *C. glabrata* RSKK 04019 and *C. albicans* ATCC 10231, which exhibited visually large inhibition zones, was also found to be insignificant ($P > 0.05$). The FCLWE was biologically active on the test microorganisms.

In a study, the antimicrobial activity of an essential oil obtained from the leaves of FC was determined by the disk diffusion method. An inhibition zone of 11 mm in diameter was observed when using *S. aureus* [33]. In our study, the FCLWE exhibited a similar level of antimicrobial activity on *S. aureus* ATCC 25923 (11.57 mm). El Hawary et al. determined the antimicrobial activity of the essential oil obtained from the leaves of

Table 1: Diameters of the inhibition zone of FCLWE using different pathogens

Pathogens	Diameter of the Inhibition Zone (mm±SD)		
	FCLWE	AM	FCA
<i>S. aureus</i> ATCC 25923	11.57±0.69 ^a	21.04±0.80	ND
<i>L. monocytogenes</i> ATCC 7644	7.06±1.23 ^b	29.57±0.10	ND
<i>C. glabrata</i> RSKK 04019	9.95±0.51 ^a	ND	20.35±0.10
<i>C. albicans</i> ATCC 10231	10.83±0.19 ^a	ND	21.85±1.76

* FCLWE: Fingered Citron Leaf Water Extract, AM: Ampicillin, FCA: Fluconazole, ND: Not Determined.

* Different superscript letters indicate significant differences ($P < 0.05$) determined by One-Way ANOVA followed by Tukey's post-hoc test.

FC using the well diffusion method. FC oil did not produce a zone of inhibition against *S. aureus* ATCC 6538 whereas a 15 mm zone of inhibition was observed *C. albicans* NRRL Y-477 [34]. In our study, the FCLWE exhibited a good degree of antibacterial activity against *S. aureus* ATCC 25923 but a lower level of antifungal activity against *C. albicans* ATCC 10231. Differences in the growing conditions of the plant, the method of extraction and the technique used to measure the antimicrobial activity may cause variations in the results.

The antimicrobial activity of FCLWE against LAB strains were determined using the disk diffusion assay and microdilution methods. The obtained inhibition zones are given in Table 2. The inhibitory activity of the FCLWE on *L. fermentum* MA-7, *L. fermentum* MA-8 and *L. vaginalis* MA-10 was immeasurable at a concentration of 0.5 mg/disk. The results of the statistical analysis showed that no significant difference was observed between the averages of *L. fermentum* MA-8 and *L. vaginalis* MA-10 with *L. fermentum* MA-7 ($P>0.05$), however, visual data indicates that the smallest inhibition zone was obtained in *L. fermentum* MA-7 at a concentration of 2 mg/disk. According to these results, the probiotic candidate *L. fermentum* MA-7 is the least inhibited or not inhibited by FCLWE.

The MIC and MBC or MFC results of the clinical, foodborne and yeast pathogens are given in Table 3. The MIC and MBC or MFC values of the FCLWE varied from 10 to 40 $\mu\text{g}/\mu\text{l}$. The minimum MIC and MBC values of the FCLWE were 10 $\mu\text{g}/\mu\text{l}$ against *S. aureus* ATCC 25923, while that of the MFC values was 20 $\mu\text{g}/\mu\text{l}$ against *C. glabrata* RSKK 04019. The results of the biological activity assays indicated that the FCLWE exhibits bactericidal and fungicidal effects against all the pathogenic microorganisms tested at various concentrations.

In a study by Akarca and Baytal, the MIC and MBC values of extracts obtained from fresh and dried leaves of *C. lemon* were determined. The MIC values of the extracts were recorded as 93.75 and 35.16 $\mu\text{g}/\mu\text{l}$ against *S. aureus* ATCC 6538 but 140.63 and 46.88 $\mu\text{g}/\mu\text{l}$ against *L. monocytogenes* ATCC 51774, respectively. The MBC values of the extracts were recorded as 39.06 and 23.44 $\mu\text{g}/\mu\text{l}$ against *S. aureus* ATCC 6538 but 93.72 and 31.25 $\mu\text{g}/\mu\text{l}$ against *L. monocytogenes* ATCC 51774, respectively [35]. In our study, the MIC and MBC values of the FCLWE on *S. aureus* ATCC 25923 and *L. monocytogenes* ATCC 7644 were found to be lower than in the referred study.

The MIC and MBC values of the FCLWE against the tested LAB are given in Table 4 which varied from 10 to 40 $\mu\text{g}/\mu\text{l}$. The MIC value of the FCLWE was 10 $\mu\text{g}/\mu\text{l}$ against all the tested bacteria, while the MBC value was 20 $\mu\text{g}/\mu\text{l}$ against *L. fermentum* MA-7.

In the current study, the SPF value of the FCLWE was determined to be 25. Due to its high value, the SPF of the cream-FCLWE mixture at different concentrations was also examined. The SPF values of the cream-FCLWE mixtures are given in Figure 2. The highest SPF of the cream-FCLWE mixture was 12.26 at an amount of 10 ml. The results of this study examining the SPF values

of the cream-FCLWE mixture at different FCLWE concentrations show that FCLWE significantly increases the SPF of the commercial cream, even at the lowest concentration.

Vella et al. determined the SPF values of essential oils obtained from the shells of various *Citrus* species

Table 2: Diameters of the inhibition zones of FCLWE against LAB

Lactic Acid Bacteria	Diameter of the Inhibition Zone (mm \pm SD)		
	FCLWE		
	0.5 mg/disk	1.0 mg/disk	2.0 mg/disk
<i>L. fermentum</i> MA-7	ND ^a	6.32 \pm 0.20 ^a	9.22 \pm 0.31 ^a
<i>L. fermentum</i> MA-8	ND ^a	6.28 \pm 0.13 ^a	10.34 \pm 0.34 ^a
<i>L. gasseri</i> MA-1	6.17 \pm 0.14 ^b	6.68 \pm 0.63 ^a	10.66 \pm 0.28 ^b
<i>L. delbrueckii</i> MA-9	6.15 \pm 0.11 ^b	6.87 \pm 0.22 ^a	11.73 \pm 0.32 ^b
<i>L. vaginalis</i> MA-10	ND ^a	6.66 \pm 0.17 ^a	10.01 \pm 0.96 ^a

*FCLWE: Fingered Citron Leaf Water Extract, ND: Not Determined.
*Different superscript letters indicate significant differences ($P<0.05$) using the One-Way ANOVA followed by Tukey's post-hoc test.

Table 3: MIC and MBC or MFC of FCLWE against pathogens

Pathogens	FCLWE	
	MIC ($\mu\text{g}/\mu\text{l}$)	MBC or MFC ($\mu\text{g}/\mu\text{l}$)
<i>S. aureus</i> ATCC 25923	10	10
<i>L. monocytogenes</i> ATCC 7644	20	40
<i>C. glabrata</i> RSKK 04019	20	20
<i>C. albicans</i> ATCC 10231	40	40

*FCLWE: Fingered Citron Leaf Water Extract, MIC and MBC or MFC: Minimum Inhibitory and Minimum Bactericidal or Fungicidal Concentrations

Table 4: MIC and MBC of the FCLWE against LAB

Lactic Acid Bacteria	FCLWE	
	MIC ($\mu\text{g}/\mu\text{l}$)	MBC ($\mu\text{g}/\mu\text{l}$)
<i>L. fermentum</i> MA-7	10	20
<i>L. fermentum</i> MA-8	10	20
<i>L. gasseri</i> MA-1	10	20
<i>L. delbrueckii</i> MA-9	10	20

*FCLWE: Fingered Citron Leaf Water Extract, MIC and MBC or MFC: Minimum Inhibitory and Minimum Bactericidal Concentrations

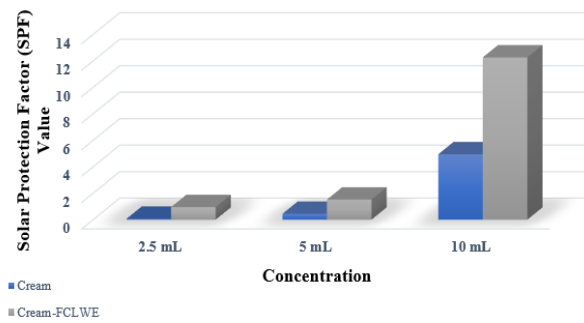


Figure 2: SPF values of the cream-FCLWE mixtures

using the Mansur equation. The obtained SPF values were presented as 2.04 for *C. limon*, 3.75 for *C. reticulata*, 8.96 for *C. aurantium* and 9.74 for *C. bergamia* [36]. The SPF of FCLWE measured by Vella et al. was higher than recorded in this study.

A cream formulation was developed by adding *L. fermentum* MA-7 sonicated in the FCLWE. In order to be effective, the cream must be reliable. The use of lysates or inactivated microorganisms reduces safety issues such as shelf life, translocation and infection, thereby increasing their usability in the cosmetics and pharmaceutical industries [37]. Additionally, *L. fermentum* MA-7 lysate used in the cream formulation is associated with antimicrobial activity and exhibited the smallest inhibition zone in the FCLWE.

The antimicrobial activities of the cream formulation developed with the commercial cream, FCLWE and probiotic were determined using the well diffusion method and the inhibition zones are given in Table 5. In the C test sample group, the diameter of the inhibition zone against the pathogenic strains (except for *C. albicans* ATCC 10231) could not be determined. The largest diameters of the inhibition zones in the case of the CW and CWP test sample groups were 2.72 and 5.00 mm against *S. aureus* ATCC 25923, respectively. The developed cream formulation may be used as an antimicrobial agent against contaminations of *S. aureus* ATCC 25923. When C, CW and CWP groups were compared statistically, a significant difference was determined for *S. aureus* ATCC 25923 and *L. monocytogenes* ATCC 7644 ($P < 0.05$). In the CP group, the diameters of the inhibition zones were determined against all the tested microorganisms (except for *L.*

monocytogenes ATCC 7644). The statistical analysis indicated that the difference between the antimicrobial activity of the C (2.08 mm) and CW (2.47 mm) groups on *C. albicans* ATCC 10231 was insignificant ($P > 0.05$), but a significant difference was observed between the CP (4.01 mm) and CWP (4.54 mm) ($P < 0.05$) groups. Common fungal infections such as *C. albicans* cause systemic infections by affecting the skin and mucosal surfaces [38]. For this reason, the developed cream formulation can provide a natural solution by exhibiting an inhibitory effect against *Candida* spp. Most importantly, an increase in the diameter of the inhibition zones was observed when comparing the C and CWP groups against all pathogen strains. Determining the biological effect of the cream formulation comprised of natural ingredients against the pathogenic strains may potentially facilitate its application in the cosmetics and pharmaceutical industries.

Salako et al. determined the effects of extracts derived from the bulb *Allium sativum* (ASB) and *C. sinensis* peel (CSP) using ethanol as a solvent on *C. albicans*. The antifungal properties of the combinations obtained by blending these extracts at concentrations of 50, 100 and 200 mg/ml and ratios of 1:1, 1:2 and 2:1 were determined by the agar well diffusion method. The ASB:CSP mixture (1:2) exhibited the highest inhibitory activity with a diameter of the inhibition zone of 21.00 mm when tested at a concentration of 50 mg/ml. This specific ASB:CSP mixture (1:2) at a concentration of 50 mg/ml was subsequently formulated into an ointment [39]. In our study, it is evident that the cream formulation containing FCLWE and probiotic provides significant protective effects against pathogens. Additionally, the cosmetics and pharmaceutical industries can develop innovative products such as creams and ointments with these formulations containing natural alternative ingredients.

4. Conclusions

The antimicrobial activity of FCLWE against clinical and foodborne pathogens as well as LAB was investigated to determine its possible applications in the pharmaceutical and cosmetics industries. It was determined that FCLWE exhibits antimicrobial and antifungal activities. FCLWE and cream formulations at different concentrations can be

Table 5: Diameters of the inhibition zones of the FCLWE and probiotic cream mixture against pathogens

Pathogens	Inhibition Zone Diameter (mm±SD)			
	C	CP	CW	CWP
<i>S. aureus</i> ATCC 25923	ND ^a	1.25±0.10 ^a	2.72±0.10 ^b	5.00±1.08 ^c
<i>L. monocytogenes</i> ATCC 7644	ND ^a	ND ^a	1.03±0.00 ^b	2.56±0.53 ^c
<i>C. glabrata</i> RSKK 04019	ND ^a	2.21±0.44 ^b	ND ^a	2.81±0.48 ^c
<i>C. albicans</i> ATCC 10231	2.08±0.10 ^a	4.01±1.16 ^b	2.47±0.09 ^a	4.54±0.70 ^c

*C: Cream (Control), CP: Cream-Probiotic, CW: Cream-Water Extract, CWP: Cream-Water Extract-Probiotic, ND: Not Determined

*Different superscript letters indicate significant differences ($P < 0.05$) by applying the One-Way ANOVA followed by the Tukey's post-hoc test.

used as alternative additives to chemical preservatives due to their high SPF values. FCLWE combined with a probiotic and commercial cream exhibited a high level of antimicrobial activity against clinical and foodborne pathogens. As a result, the water extract from FC leaves could be pioneering as far as the development of new plant-based products in both the cosmetics and pharmaceutical industries is concerned to protect the skin against pathogens as well as provide an alternative to chemical preservatives that threaten public health.

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