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Antibacterial Activity of Lemon IntegroPectin Against *Escherichia coli*

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Lemon IntegroPectin sourced via hydrodynamic cavitation of lemon industrial processing waste in water only shows high antibacterial activity against Gram-negative *Escherichia coli*. Insight on the antibacterial mechanism gained by investigating the oxidation kinetics of the phytocomplex at 0.5 mg/mL concentration in contact with the bacteria indicates that the oxidative stress, measured by reactive oxygen species (ROS) produced at the bacterial surface, is significantly higher than that generated by H_2O_2 0.5 M. Given the nontoxic and mitoprotective nature of this bioconjugate sustainably extracted from an abundant biological resource, these findings are promising toward a new antimicrobial treatment against polymicrobial infections unlikely to develop drug resistance.

1. Introduction

Escherichia coli is a versatile Gram-negative bacterial strain colonizing the human gastrointestinal tract, where it becomes part of the microbiota a few hours after birth. Specific pathotypes of *E. coli* may infect healthy individuals causing enteric disease and diarrhea, urinary tract infection, and meningitis.[1] Multidrug-resistant *E. coli* is a serious threat to human health and to the environment due to dissemination of antibiotic resistance genes.[2] Originally employed since the 1940s as antibiotic against human and animal infections caused by Gram-negative bacteria in hospitals and animal farms, polymyxin E (colistin) was first discontinued due to significant toxicity.[3] However, the significant increase in the frequency of multidrug resistant (MDR) Gram-negative bacteria such as *E. coli* and the limited availability of novel antibiotics has recently led to resume the use of colistin as a last-resort antibiotic.[4,5]

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The search of antimicrobials capable to prevent the emergence of multidrug-resistance is an active field of research in many countries. Research is focused on nontraditional approaches to combat MDR, including the development of quorum sensing (QS)-targeted therapy, bacteriophage therapy, antimicrobial peptides, and nanoparticle-based therapy.[6]

The use of phytochemicals with antimicrobial activity isolated from plants, fungi, animals, and mushrooms to combat MDR is particularly promising and intensively researched[7] because natural products are less likely to drive antimicrobial resistance due to mechanisms that often involve interference with QS and bacterial membrane disruption.

Originally reported in the late 1930s, the antibacterial properties of pectin were rediscovered in the late 1990s.[8] For instance, pectin, a complex heteropolysaccharide commercially derived from citrus peel or apple pomace, is the only food fiber showing antibacterial activity, decreasing the need for antimicrobial penicillin.[9]

In 2014, scholars in Taiwan reported lack of antibacterial activity of citrus pectin against *Pseudomonas aeruginosa*.[10] In 2021, however, along with Presentato and others, we reported that lemon IntegroPectin shows bactericidal activity against *P. aeruginosa* with minimum bactericidal concentration (MBC) of 15 mg/mL.[11] For comparison, the MBC of commercial citrus pectin derived from dried lemon peel was 40 mg/mL, nearly three times higher.[11] The strain tested was *P. aeruginosa* 10145, a standard indicator pathogen strain commonly used as a quality control strain for drugs.[12]

Lemon IntegroPectin is a new pectin-based phytocomplex reproducibly sourced via hydrodynamic cavitation of lemon industrial processing waste carried out in water only directly on semi-industrial scale. In detail, 34 kg of lemon processing waste from an in-line extractor at a lemon juice factory processing organically grown lemons were suspended in 120 L tap water, using the same cavitation conditions developed in 2019 to process waste orange peel.[13] Readily dissolved in water, the resulting yellow pectin flakes obtained after freeze dry-

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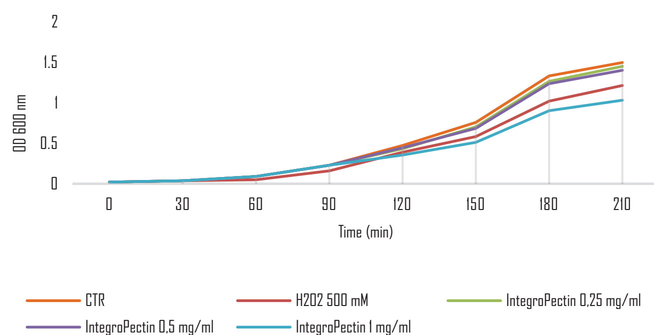

Figure 1. Effect of lemon IntegroPectin increasing concentrations on *E. coli* proliferation. CTR stands for control sample, and H_2O_2 500 mM represents the positive control of oxidative stress.

ing are completely devoid of cytotoxic activity against human pulmonary cells up to high concentration (1 mg/mL)[14] and exert a powerful antioxidant and mitoprotective activity on human neuronal cells.[15] We now report the high in vitro inhibitory activity of lemon IntegroPectin against *E. coli* bacterial strain.

2. Results and Discussion

To establish the antimicrobial activity, we measured the optical density at 600 nm (at this wavelength the bacterial cells are not harmed, whereas light scattered by the cells no longer reaches the photoelectric cell translating into higher turbidity) of the challenged bacterial culture as compared to unchallenged cells.

Figure 1 shows evidence of the increasing antibacterial activity of lemon IntegroPectin at increasing concentrations against *E. coli* in Luria Bertani medium challenged with increasing concentrations (i.e., 0.25, 0.5, and 1.0 mg/mL) of lemon IntegroPectin at 37 °C under mechanical shacking.

Although little activity was observed at 0.25 mg/mL concentration, it was enough to add the newly extracted lemon IntegroPectin at 1.0 mg/mL concentration to observe a dramatic reduction in the *E. coli* cell proliferation, significantly higher than that caused by concentrated (0.5 M) aqueous hydrogen peroxide (H_2O_2). Hydrogen peroxide is a powerful oxidizing agent that at said concentration is capable to quickly denature enzymes and oxidize not only protein side chains but also the protein backbone.[16]

To evaluate the possible generation of reactive oxygen species (ROS), *E. coli* bacteria were incubated with a diluted solution of dichlorodihydrofluorescein diacetate (DCFH-DA), the most widely used probe for detecting intracellular H_2O_2 and oxidative stress,[17] alone or in the presence of increasing concentrations of lemon IntegroPectin.

Figure 2 shows that already at 0.5 mg/mL concentration, the phytocomplex exerts a powerful oxidative stress on the bacteria, significantly higher than H_2O_2 0.5 M. When the pectin concentration was increased to 1.0 mg/mL, ROS generation became more than twice higher than that driven by concentrated H_2O_2. Figure 3, in turn, demonstrates the quick production of ROS, esti-

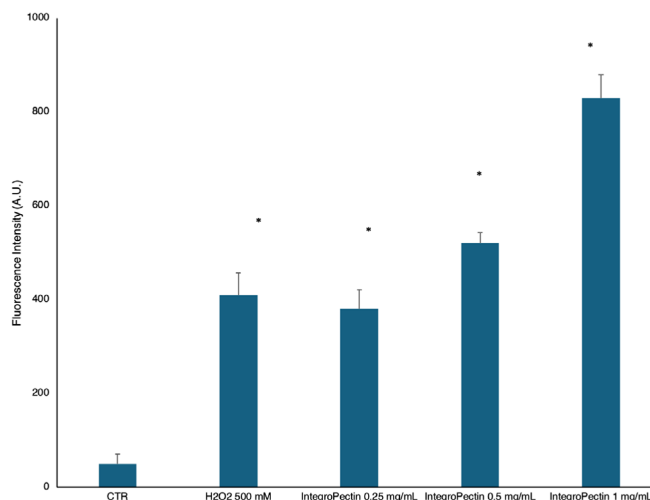

Figure 2. Increase in oxidative stress on E. coli bacteria induced by lemon IntegroPectin at increasing concentrations. 0.5 M aqueous H_2O_2 as a positive control for oxidative stress. CTR stands for control sample. (*p-value < 0.1).

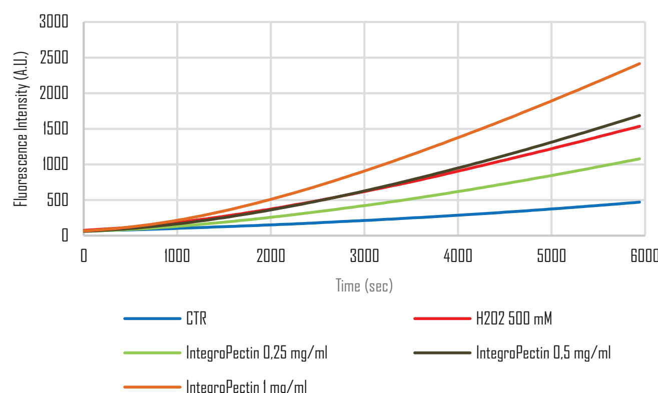

Figure 3. *E. coli* oxidation kinetics driven by H_2O_2 0.5 M and by increasing concentrations of lemon IntegroPectin. CTR stands for control sample.

mated using DCFH-DA, after increasing the lemon IntegroPectin concentration from 0.25 to 1 mg/mL.

ROS generation at the surface of *E. coli* followed an almost identical kinetic profile for both H_2O_2 500 mM and aqueous IntegroPectin at 0.5 mg/mL concentration. Yet, using lemon IntegroPectin at 1.0 mg/mL, the amount of ROS after 16 min (~1000 s) was higher than that generated by concentrated hydrogen peroxide. Eventually, after 1.7 h, the amount of ROS generated by addition of the new pectic substance was almost double when compared to the oxidative stress exerted by H_2O_2 0.5 M.

Lemon IntegroPectin has an extremely high content of polyphenols adsorbed at its surface: 0.88 mg GAE/g (in terms of gallic acid equivalents or GAE per dry gram of pectin) versus 8.3×10^{-3} mg GAE/g for the lemon peel of the cultivar with the highest biophenol concentration.[18]

Studying in detail the controlled release of bioactive citrus flavonoids and terpenes adsorbed and concentrated at the lemon IntegroPectin surface, along with Alduina et al., we found that this new bioconjugate chiefly releases flavonols such as kaempferol, phenolic acids (*p*-coumaric acid and gallic

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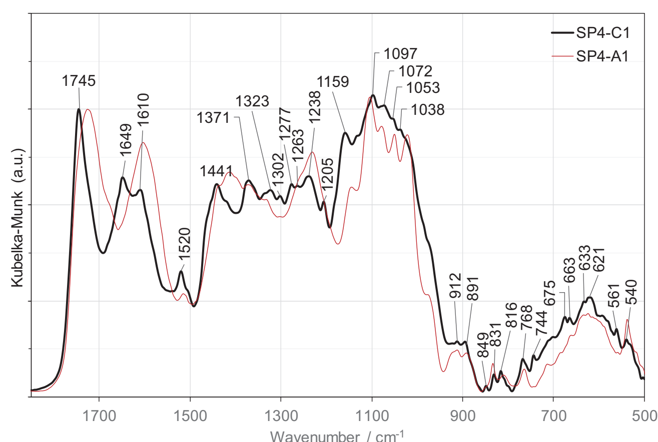

Figure 4. Comparison of the DRIFT spectra of commercial lemon pectin (sample SP4-A1) and lemon IntegroPectin, SP4-C1.

acid), and monoterpenoid safranal via a quasi Fickian diffusion mechanism.[19]

The antibacterial action of lemon IntegroPectin is therefore due to a synergistic mechanism, involving the intrinsic antibacterial activity of the pectic polymer—characterized with its unique structure showing a low degree of methylation and abundant RG-I regions when compared to conventional citrus pectin—and the antibacterial activity of the aforementioned citrus flavonoids and terpenes at the surface of the IntegroPectin.[11,19]

Both gallic acid[20] and kaempferol[21] in contact with bacterial cells generate ROS through different mechanisms (kaempferol through the interaction of its phenoxyl radical with oxygen, and gallic acid upon photooxidation reacting with oxygen), whereas *p*-coumaric acid kills bacteria through a dual damage mechanism involving disruption of outer and plasma membrane permeability and binding to bacterial genomic DNA.[22] Safranal, in turn, binds and inhibits *E. coli* ATP synthase and cell growth.[23]

Compared with commercial citrus pectin sourced from dried lemon peel using hot mineral acid followed by precipitation with alcohol,[24] lemon IntegroPectin has a higher proportion of type I rhamnogalacturonan, containing side-chains of α-1,5-l-arabinosyl and β-1,4-d-galactosyl residues and a low degree of methylation (DM, the percent of methyl-esterified carboxyl groups) of 27%,[15] much lower than 69% DM found in commercial lemon pectin employed used in comparative tests.

Figure 4 compares the spectrum of the aforementioned lemon commercial pectin (SP4-A1) with that of the corresponding IntegroPectin (SP4-C1). The 4000–2100 cm^{-1} region was normalized to the hydroxyl stretching band, and the 1850–500 cm^{-1} region was normalized to the carbonyl stretching band. Clearly, the spectrum of lemon IntegroPectin is very different from that of commercial citrus pectin, with the C—H stretching bands very well defined.

Besides, the important shifts in the carbonyl and C═C stretching bands, with two very well-defined bands at 1649 and 1610 cm^{-1}, along with changes in the relative intensities in the 1500–900 cm^{-1} range, suggest the presence of aromatic compounds and other essential oils' components, such as polymethoxyflavones and other flavonoids, in lemon IntegroPectin.

3. Conclusions

In conclusion, investigating the antibacterial mechanism of lemon IntegroPectin against *E. coli*, we found that the phytocomplex, when dissolved in the culture broth at the concentration of 1 mg/mL, exerts a powerful oxidative stress action against *E. coli*, more than twice as strong as concentrated (0.5 M) hydrogen peroxide.

Considering that lemon IntegroPectin exerts significant antibacterial activity in vitro against *S. aureus* virulent strains,[11] as well as its lack of cytotoxicity against human lung epithelial cells[14] and its protective action exerted on human neuronal cells at the aforementioned concentration (1 mg/mL),[15] these findings are promising toward a new antimicrobial treatment against polymicrobial infections, which are unlikely to develop MDR. The multitarget mechanism of action indeed involves attachment of the low-methoxyl IntegroPectin to the bacterial membrane, followed by the sustained (prolonged) release of its high content of bioactive citrus flavonoids and terpenes.[19] It is encouraging, in this respect, that the antibacterial mechanism of flavonoids against multidrug resistant Gram-positive bacteria (and as adjuvants of colistin against MDR Gram-negative pathogens), recently identified by Sheng et al., relies on a membrane-disrupting mechanism against. This approach reduces the likelihood of bacteria developing antibiotic resistance as bacterial membrane can barely change without loss of function.[25]

4. Experimental Section

4.1. Experiments with *E. coli*

Lemon IntegroPectin was sourced by hydrodynamic cavitation of organically grown lemon processing waste carried out in water only, followed by freeze-drying of the aqueous extract, as previously reported.[13,14] Industrial lemon processing waste was kindly donated by a citrus company in Sicily (OPAC Campisi, Siracusa, Italy).

A single colony was inoculated into LB medium and incubated at 37 °C overnight (o.n.). An aliquot (5 µL) of the o.n. bacterial culture, approximately 109 CFU/mL, was added to three test tubes containing fresh LB medium (5 mL). Lemon IntegroPectin (0.25, 0.5 =, and 1 mg/mL) was added separately to the culture medium at $t = 0$ min or $t = 210$ min. The growth was determined by reading the absorbance value at 600 nm (OD 600) using a Spark 10 M multimode microplate reader (Tecan Group, Männedorf, Switzerland) with 30 min intervals.

For ROS generation, an aliquot of the *E. coli* o.n. culture, approximately 109 CFU/mL, was diluted (1:105), and a 100 µL sample was placed in a 96-well optical bottom white microplate. Lemon IntegroPectin at different concentration (0.25, 0.5, and 1 mg/mL) was added to the wells. Then, the samples were incubated with 1 mM of DCFH-DA (Molecular Probes, Eugene, OR, USA) for 8 h at 37 °C. Afterward, the *E. coli* samples were analyzed using a GloMax Microplate Reader (Promega, Madison, WI, USA) for fluorescence detection at the excitation wavelength of 475 nm and emission wavelength

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555 nm. An untreated *E. coli* bacterial culture (CTR) and an *E. coli* culture challenged with 500 mM H_2O_2 were used as control for growth and oxidative stress.

For the oxidation kinetic measurements, an aliquot of *E. coli* o.n. culture, approximately 109 CFU/mL, was diluted (1:105), and a 100 µL sample was placed in a 96-well optical bottom white microplate. Lemon IntegroPectin at different concentration (0.25, 0.5, and 1 mg/mL) was added to the wells. The production of ROS was estimated using DCFH-DA at a final concentration of 1 mM. Untreated *E. coli* (CTR) was used as control, and aqueous H_2O_2 0.5 M was used as a positive control of oxidation state. The oxidation kinetics was followed by fluorescence at the excitation wavelength of 475 nm and emission wavelength 555 nm by a Glo-Max Discover System (Promega, Madison, WI, USA) in a 96-multiwell plate incubated for 2 h at 37 °C.

4.2. Lemon IntegroPectin Structural Characterization by DRIFT

The molecular structure of pectin samples, commercial lemon pectin (citrus pectin purchased by Sigma-Aldrich, Milan, Italy, DM = 69%, galacturonic acid > 74.0%, dried basis), and lemon IntegroPectin was characterized by diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy, using a Mattson RS1 FTIR spectrometer equipped with a wide band MCT detector and a Specac selector, in the of 4000–500 cm^{-1} range at 4 cm^{-1} resolution. The spectra were the result of ratioing 500 coadded single beam scans for each sample (grinded pectin powder diluted in grinded FTIR grade KBr, in the appropriate proportion to assure the validity of the Kubelka–Munk assumptions)[26] against the same number of scans for the background (grinded KBr). The spectra were converted to Kubelka–Munk units and further processed using Microsoft Excel.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords: Antibacterial · Citrus · *Escherichia coli* · Flavonoids · IntegroPectin

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