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SHORT COMMUNICATION

Proteins of the fruit pulp of *Acca sellowiana* have antimicrobial activity directed against the bacterial membranes

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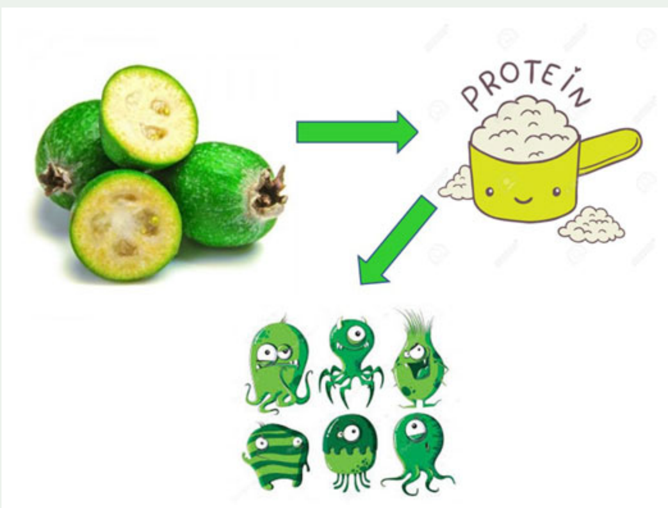
ABSTRACT

Acca sellowiana is an evergreen plant that produces edible fruit whit high nutritional properties, and also contains clinically relevant bioactive compounds, including polyphenols and essential oils. Numerous biological activities were demonstrated for *A. sellowiana* fruits: antifungal and antitumoral and anti-oxidant. It was also showed a strong antibacterial activity against Gram-positive and Gram-negative bacteria. Fruit are generally considered recalcitrant plant tissues for the difficulty to obtain high quality protein due to a low protein content and the presence of interfering substances. For this reason, the objective of the present work was obtain high quantity of protein extract, to determine the location in the fruit of the molecules responsible for the antibacterial activity, separate them according to molecular weight, test their thermo resistance, study the timing of action, isolate the protein fraction having activity and hypothesise a mechanism of action directed against bacterial membranes.



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1. Introduction

Acca sellowiana (Mytraceae) is an evergreen shrub native of South America that produces edible fruit whit an high nutritional value (Romero-Rodriguez et al. 1994). *A. sellowiana* also contains clinically relevant bioactive compounds, including polyphenols and essential oils (El-Shenawy et al. 2008; Basile et al. 2010a; Zhu 2018). Numerous biological activities were demonstrated for *A. sellowiana* fruits: antioxidant, *in vitro* on human blood cells, on gastric cells and *in vivo* in rats (Ielpo et al. 1997; Vuotto et al. 2000; Rossi et al. 2007; Turco et al. 2016; Piscopo et al. 2018), antifungal against human and phytopathogenic fungi (Basile et al. 2010b) and antitumoral in numerous cancer models (Bontempo et al. 2007; Turco et al. 2016; Dell'Olmoa et al. 2019). It was also showed a strong antibacterial activity for *Acca sellowiana* fruit (Basile et al. 1997) demonstrating also that flavones isolated from *A. sellowiana* fruit inhibits Gram-positive and Gram-negative bacteria (Basile et al. 2010b). Recently it was demonstrated that acetonic extract from *A. sellowiana* fruit inhibited bacterial biofilm formation of both sensitive and resistant bacterial strains. Treatment with fruit extract induced a dramatic decrease of cell cohesion, whereas no significant increase in cell death was observed (Dell'Olmoa et al. 2019). Peptides and proteins are promising sources of anti-microbial and anti-oxidant compounds (Maróti et al. 2011). Fruit are generally considered recalcitrant plant tissues for the difficulty to obtain high quality protein due to a low protein content and the presence of interfering substances such as polyphenols, carbohydrates, polysaccharides, pigments and starch (Sarmadi and Ismail 2010). Protein fraction obtained from *A. sellowiana* fruit showed antibacterial activity against different Gram-positive and Gram-negative bacteria both as American type culture collection (ATCC) standard and clinically isolated strains. For this reason, the objective of the present work was: to determine the location in the fruit of the molecules responsible for the antibacterial activity, separate them according to molecular weight, test their thermo resistance, study the timing of action, isolate the protein fraction having activity and hypothesise a mechanism of action directed against bacterial membranes.

2. Result and discussion

2.1. Antimicrobial activity, heat resistance and separation by molecular size of the Acca sellowiana fruit acetone extracts

In order to characterise the antimicrobial activity of total fraction extracted from the *A. sellowiana* fruits, we have carried out Kirby and Bauer assays. The antimicrobial activity of different fractions is shown in Supplementary information Figure S1: the total fraction was active against both *Escherichia coli* and *Pseudomonas aeruginosa* strains and it was particularly active against *P. aeruginosa* PAOI. In order to investigate the temperature resistance of the molecule responsible for the antimicrobial activity, we heated the total extract up to a temperature of 65 °C. As shown in Supplementary information Figure S1, the heated extracts show the same antimicrobial activity of the control. At the same time to have indication on the molecular weight of the active molecules, the experiment was repeated by fractionating the total extract with filters having a cut-off of 3 kDa. The two fractions, containing molecules bigger or smaller than 3 kDa were used to measure the antimicrobial activity which is still present in both fractions as is possible to see in Supplementary information Figure S1. In order to analyse the shape of bacterial cells after treatment with Acca extracts, we performed TEM observations showing that the untreated control of *P. aeruginosa* cells (Supplementary information Figure S2A), or negative control (Supplementary information Figure S2B) have standard features of bacterial cells (electron dense protoplasm surrounded by a very electron dense cell wall with a solid texture). Treatments with Acca fraction <3kDa or >3kDa (Supplementary information Figure S2C, S2D), induced the appearance of altered bacteria with electron clear or even almost empty protoplasm delimited by still well visible, electron dense cell walls. Several literature studies (Basile et al. 2001; 2010b) show that the antimicrobial activity of *A. sellowiana* extracts depends in part on a chemical compound called flavone belonging to the flavonoid class. The weight of the flavone is 222.24 Da and probably it is contained in the minor fraction of 3 kDa. The next step was to compare the antimicrobial activity of the commercial flavone at a final concentration of 1 μg/mL with the low molecular weight fraction at five times concentration value (5 μg/mL). In the Supplementary information Figure S3 is evident that the two samples behave in a very similar manner against both bacterial strains, moreover both the flavone and the minor fraction of 3 kDa have the maximum activity after 120 min from incubation, confirming that the activity contained in the minor fraction of 3 kDa is probably due also to the presence of the flavone.

2.2. Antimicrobial properties of the Acca sellowiana pulp

According to other studies (Basile et al. 1997) the flavone is mainly contained in the peel of the *A. sellowiana* fruits, so we decided to separate the peel from the rest of the fruit by performing an acetonic extraction on both parts of Acca. As it can be seen in Supplementary information Figure S4 panel A, both the skin and the pulp in which the seeds are immersed show good antimicrobial activity. The pulp activity is greater than that of the peel. Because we are interested in new molecules, having antimicrobial activity, we performed protein extraction from pulp (including seeds). As shown in Supplementary information Figure S4, the protein extract, first loaded onto polyacrylamide gel under denaturing conditions (panel B) and then tested on *P. aeruginosa* (panel C), has a good antimicrobial activity, and as the protein extract concentration increased (from 0 to 100 μg/mL) the bacterial mortality increased too, until the death of all bacterial cells. To preliminary understand the antimicrobial mechanism of action of the extracted protein fractions we performed an experiment to evaluate the interaction between the pulp protein and the *P. aeruginosa* strain using DAPI as DNA fluorescent stain and the fluorescent PI dye that is only able to penetrate damaged membranes and is generally excluded from viable cells. Our results, obtained using 50 μg/mL of pulp protein fraction, showed that almost all the treated cells emit red fluorescence light as a consequence of an impairment of membrane integrity (Supplementary information Figure S5D). As expected, fluorescence of untreated cells was blue because PI could not cross their intact plasma membranes (Supplementary information Figure S5B). Other experiments were conducted in order to determine the cytotoxicity by MTT assays of all the samples used, the experiments were performed using different concentrations of flavone, acetone extract and protein extracts of pulp and peel, incubated for 24 and 48 h (see methods). All the tested molecules did not show toxicity in the MTT assay (Data not shown).

3. Conclusion

Several compounds are known which are isolated from the acetone extracts of the Acca fruit, but proteins or peptides able to inhibit growth or to kill bacterial strains, even pathogens such as *P. aeruginosa*, have not been identified. In this article, we demonstrate that *A. sellowiana* fruit is effective in preventing or killing *E. coli* and *P. aeruginosa* cells. The proteins isolated from the pulp seem to have good antimicrobial activity, they probably act by damaging the bacterial membranes. Moreover experiments are underway to determine which proteins have antimicrobial activity within the protein mixture. The extract of this fruit having already nutraceutical and antimicrobial properties seems very promising.

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Disclosure statement

Authors declare no conflict of interests.

Author contributions

All authors planned and carried out the experimental work, discussed the results and commented on the manuscript.

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