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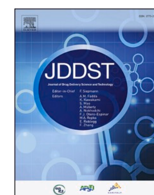

Design of advanced buccal films with kiwiberry extract to prevent oral mucositis: From *in vitro* buccal models to *ex vivo* studies

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A R T I C L E I N F O

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A B S T R A C T

Oral mucositis (OM) is a common side effect of cancer treatments characterized by disruption of the oral mucosa integrity, inflammation, and pain. The treatment strategies to prevent and treat OM are still unsatisfactory, leading to the search of new active compounds, particularly from natural sources, such as *Actinidia arguta* fruits. *A. arguta* is a perennial vine and its fruit, commonly known as kiwiberry, has been associated with different therapeutic properties and pro-healthy benefits, particularly antioxidant, anti-inflammatory and anticancer effects. These bioactive properties are due to the fruit outstanding content in phenolic compounds, vitamins, and organic acids, which attracted the researcher's attention for potential application in pharmaceutical industry. The aim of this study is to develop buccal films with *A. arguta* fruit extract as active ingredient to prevent OM symptoms. The films were prepared by solvent casting after placing 50 g of a buccal film mixture prepared with 1% of HPMC K100 LV EP, 2.5% glycerin, and 100 mL of *A. arguta* extract as solvent. Different films parameters were assessed, namely physical features (weight: 194.8 mg; thickness: 0.37 mm; disintegration time: 15.05 min; moisture content: 10.53%; swelling capacity: 55.95%), mechanical properties (resistance to extension: 10.11 N; percent of elongation: 36.10%; Young's modulus: 0.0034 MPa) and antioxidant/antiradical activities (TPC = 6.46 mg GAE/g film; FRAP = 49.45 μmol FSE/g film; ABTS = 3.74 mg AAE/g film; DPPH = 4.90 mg TE/g film). *In vitro* cell assays attested the absence of negative effects on HSC-3 and TR146 oral cell lines. Most important, the compounds release profile were assessed through *in vitro* and *ex vivo* models coupled to LC/DAD-ESI-MS quantification and the results revealed high permeation of rutin, quercetin-3-*O*-glucoside and catechin. Overall, these results highlight the significant potential of buccal films with *A. arguta* fruit extract to prevent OM condition.

1. Introduction

Cancer is the leading cause of death worldwide, with more than 19.3 million new diagnoses and approximately 10 million deaths in 2020 [1]. The aggressiveness of the treatments employed to solve or delay the disease progression and promote the patients' longevity, such as radiation, chemotherapy or the combination of both, leads to the development of side effects, particularly oral mucositis (OM) [2,3]. This condition is characterized by a disruption of the oral mucosa integrity that results in pain, oral inflammation, ulcers, significant discomfort, and speaking and swallowing difficulties [3–6], conducting to severe weight loss, inability to take medications, and an overall reduction of life quality [3,5,7]. Furthermore, this loss of integrity may provide easy access for microorganisms, leading to serious complications, such as

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sepsis [5,8]. Currently, the OM treatment consists in the administration of radical oxygen species (ROS) scavenging drugs, pro-inflammatory cytokines inhibitors and prebiotics, oral care and cryotherapy, nutritional supplementation, and pain management [5,7]. However, as OM progresses, the treatment strategies become insufficient [4] and the search for new active substances with potential therapeutic properties is urgently needed. Natural matrices, such as chestnut shells [8], honey [2, 9], *Aloe vera* [10,11], chamomile and green tea [9,12], ginger [13], and *Actinidia arguta* [14], have been investigated for this purpose. Since these matrices are extremely rich in antioxidant compounds, mainly polyphenols [4], they may inhibit the prevalent pathways of OM development, such as ROS generation that results in oxidative stress [4, 7]. Additionally, natural sources may have anti-tumor activity, through direct interaction with cancer cells in early stages of development [5].

Among natural matrices, *A. arguta* fruit arises as an extraordinary candidate for OM prevention. *A. arguta* is a perennial vine that mostly grows in Asian countries [15,16], being described as a traditional herbal medicine in Korea [17]. The small smooth-skinned fruit, known as kiwiberry or hardy kiwi [16], has been associated with different therapeutic properties and pro-healthy benefits, particularly antioxidant, anti-microbial and anticancer activities [18–20]. These accomplishments are due to the outstanding content in bioactive compounds, namely vitamin C (78.30 mg/100 g fresh weight (fw)) [21] and phenolics (from 2443.30 to 6679.18 mg/100 g dry weight (dw)) [22], with chlorogenic acid, quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucoside, kaempferol-3-*O*-galactoside, caffeic acid, and quinic acid being the most prominent ones [19,20,22]. The remarkable bioactive composition of kiwiberry, coupled to increasing concerns related with byproducts valorization [8,23,24], attracted the researcher's attention for the potential reuse of kiwiberries byproducts, such as leaves and fruits without caliver to be commercialized [25,26], for the prevention of OM [8,14].

Given the complexity of the oral cavity system, divided in epithelium (thick and avascular) and underlying tissue (vascular) [27], the buccal route may be considered a suitable systemic delivery to prevent and treat OM, ensuring a quick absorption and a rapid action with reduced side effects [4,28]. In the last decades, films have emerged as a valuable drug delivery technology for this purpose [29,30]. Aside from their small size and thickness, these delivery systems are characterized by a painless application, presenting a protective barrier effect, potential for removal in case of negative therapeutic side effects, and local and/or systematic action [31,32], which increase patient compliance. Particularly, buccal films have the important benefit of allowing substances to be adsorbed without passing through the gastric tract, avoiding the action of enzymes and acids [28,33] and increasing the phenolic compounds' bioavailability [24], while simultaneously enabling the administration to vulnerable patients, such as pediatric, geriatric, and dysphasic patients [29,32,34]. Therefore, the main goal of this study was to incorporate an *A. arguta* fruit extract in buccal films to explore their potential use to prevent OM symptoms.

2. Materials and methods

2.1. Chemicals and reagents

The reagents used in the present study were acquired from Sigma-Aldrich (Steinheim, Germany) and Sigma Chemical Co. (St. Louis, USA). The LC/DAD-ESI-MS solvents were supplied by Merck (Darmstadt, Germany). The human tongue squamous carcinoma cell line (HSC-3; passage 33–38) as well as the human squamous cell carcinoma (TR146; passage 17–20) were acquired from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells reagents were supplied by Invitrogen Corporation (Life Technologies, S.A., Madrid, Spain).

2.2. A. arguta extract preparation

A. arguta fruits were supplied by Mini-Kiwi Farm (Landim, Vila Nova de Famalicão, Portugal). The samples were dehydrated (Excalibur Food Dehydrator, Sacramento, CA, USA) at 40 °C for 24 h and grinded in a miller (Moulinex A320, France). The kiwiberry extract was obtained through ultrasound-assisted extraction, using an ultrasonic probe processor (Sonic Vibracell, model VCX50, Newtown, CT, USA) with a 13 mm probe, as described by Macedo et al. [19]. The conditions used were a sample-to-solvent ratio of 1:40 g/mL, a probe amplitude of 50%, 750 W of power, 20 kHz of frequency and a hydroalcoholic solvent (50% water (H_2O):50% etanhol (EtOH)) for 17.5 min at room temperature. After extraction, the extract was filtered through a Whatman n °1 paper and kept at 4 °C overnight. Afterwards, the extract was centrifuged twice at 18 500×*g* (13 000 rpm in Megafuge™ 16, Thermo Scientific, Massachusetts, USA) for 10 min at 20 °C for debris removal and stored at 4 °C until further analysis.

2.3. Buccal films

An initial optimization of buccal films production was implemented using different polymers, such as Polyvinylpyrrolidone (PVP), Carbopol EDT 2020, Eudragit E100, Eudraguard Protect, Eudragit RS 30 D, Hydroxypropyl Methylcellulose (HPMC) K100LV EP, HPMC K4M and HPMC K100 M at different concentrations. The concentration of glycerin, employed as plasticizing agent, was also adjusted. The qualitative parameters were used for an initial evaluation, namely surface roughness, and presence of excessive glycerin residue (data not shown). After preliminary studies, glycerin was fixed to 2.5% (*w/v*), HPMC K100 LV EP solution to 1% (*w/v*) and 100 mL water or *A. arguta* fruit extract were used as solvents, respectively, for the control and the *A. arguta* fruit extract buccal films. The buccal films mixture was mixed in an ultrasonic bath (Bandelin Sonex and JP Selecta) for 1 h, and then 50 g of buccal films mixture were transferred into a petri dish (8.5 cm of diameter) and incubated at 4 °C for 24 h to remove the air bubbles. Afterwards, the petri dishes were placed in an oven (Model no. 2000208, J.P. Selecta, Barcelona, Spain), at 40 °C for 48 h, for solvent casting. Finally, the buccal films were cut into 2 × 2 cm portions (4 cm^2) and conserved in a desiccator for further analysis.

2.4. Buccal films characterization

2.4.1. Mass uniformity

The buccal films' weight was evaluated in an analytical balance (KERN ALS, Radwag and Mettler Toledo) to assess the mass uniformity. Different films portions (4 cm^2) from different batches ($n = 9$) were weighed individually and the mean weight and standard deviation were calculated.

2.4.2. Thickness

Thickness was measured using a calibrated electronic digital micrometer (MICROM-DIG, Portugal). The measurements were performed in three different sections of each buccal film portion (4 cm^2) from different batches ($n = 9$).

2.4.3. Folding endurance

Buccal film portions (4 cm^2) were repeatedly folded (180°) at the same point until break or observe signs of tare [35,36]. A limit of 300 folds was set. The number of times the films were folded at the same point was accounted as folding endurance value. A total of nine film portions were evaluated ($n = 3$).

2.4.4. Moisture content and swelling capacity

Moisture content and swelling capacity were evaluated according to Campos et al. [37], with minor modifications. Briefly, film portions (4 cm^2) from different batches ($n = 3$) were weighted and placed in an infrared moisture balance AD-4713 (A&D Company; Tokyo, Japan) with an infrared lamp heated at 80 °C for 30 min. The device automatically calculated the moisture percentage through the differential of the

sample weight before and after heating. Afterwards, new buccal film portions were hydrated for 2 min and 30 s with 20 mL PBS (pH 7.4) and gently collected by filtration with a Whatman n °1 paper to remove the excess of water. The moisture of the hydrated buccal film portions was measured as before. The increase of water mass was determined as the indirect swelling capacity.

2.4.5. Disintegration time

The disintegration time was evaluated by dipping a buccal film portion (4 cm^2) into a petri dish containing 20 mL of artificial saliva (pH 6.9) pre-heated at 37 °C and with constant stirring. Different buccal film portions from different batches were evaluated ($n = 3$). The results are presented in minutes and the time stopped when the complete disintegration occurred.

2.4.6. Scanning electron microscopy

Scanning electron microscopy (SEM) was used to analyze the surface and morphology of the produced buccal films, using a Fei Quanta 400 FEG ESEM/EDAX Pegasus X4M (Eindhoven, The Netherlands). This investigation was conducted in a JEOL JFC-100 apparatus at a 2,00 kV accelerating voltage and a 1000–5000× magnification. Samples were placed into the analyzer by using double-sided sticky tape to fixate the buccal films in a brass stub that was subsequently coated in vacuum with a thin layer of gold.

2.5. Mechanical properties

Mechanical properties, namely resistance to extension (N), percent of elongation (%) and Young's modulus (Eq. (1) and Eq. (2), respectively), were evaluated in three independent film portions ($n = 3$) using a texture analyzer (TA.XT plus Texture Analyzer, Stable Micro Systems, Cardiff, UK) equipped with the Texture Exponent 32 software (version 6.1.12.0, Stable Micro Systems; Surrey, UK). Briefly, the buccal films were cut in 4 × 1 cm portions and were vertically held using the tensile grip probe with a separation of 20 mm. Afterwards, films were stretched until rupture by moving the probe at a constant speed of 0.1 mm/s. The force and height calibrations were performed with a weight of 5 kg and Mini Tensile Grips (Stable Micro Systems), respectively.

$$Percent\ elongation\ (\%) = \frac{distance\ at\ the\ rupture\ instant - inicial\ grip\ distance}{inicial\ grip\ distance} \times 100\% \quad (1)$$

$$Young's\ modulus\ (MPa) = \frac{force\ at\ corresponding\ strain}{cross-sectional\ area\ of\ the\ film \times corresponding\ strain} \quad (2)$$

2.6. Thermal behavior

The investigation of compatibility on the formulated buccal films, the polymer used in the films, and the lyophilized extract was made with Differential Scanning Calorimetry (DSC). The experiments were performed individually, within a controlled nitrogen atmosphere with a flow rate of 40 mL/min, through a DSC 200 F3 Maia (Netzsh–Gerätebau GmbH, Germany). Samples were sealed into aluminum pans and the temperature range spanned from 20 °C to 200 °C with a heating rate of 10 °C/min using an empty aluminum pan as a reference. The onset temperatures were calculated using Proteus Analysis software (Version 6.1, Netzsh-Gerätebau GmbH, Germany).

2.7. Phytochemical composition

The phytochemical composition of the buccal films was determined by liquid chromatography equipped with triple quadruple mass spectrometry (LC/DAD-ESI-MS), according to the procedure described by Teixeira et al. [14]. The stationary phase used was an Agilent Eclipse XDB C-18 (3.0 × 150 mm) 3.5 μm column and the mobile phase consisted of a gradient of three compounds: water 1% formic (A), acetonitrile (B) and methanol (C). The gradient started at 95% A, 5% B and 0% C and continued to 0% A, 90% B and 10% C, over 30 min. The flow rate was 0.4 mL/min, with the column temperature stable at 30 °C. The sample injection volume was 20 μL. The sample was diluted 10 times in methanol:water (50:50, *v:v*) and centrifuged at 13 300 rpm for 15 min. MS spectra were recorded in negative ion mode in 150–2000 *m/z* range. For quantification purposes, the standard calibration curves used were:

Catechin curve: $y = 250231.55x + 40130.99$ ($R^2 = 1$)

Rutin curve: $y = 115677.28x - 1050.56$ ($R^2 = 1$)

The results obtained were expressed as mg of each compound per 100 g of film (mg compound/100 g film).

2.8. In vitro antioxidant and antiradical activities

2.8.1. Total phenolic content

The Folin–Ciocalteu method was used to determine the total phenolic content (TPC), following the methodology described by Singleton and Rossi [38], with minor modifications. To this end, a film portion with an area of 0.5 cm^2 was dissolved in 1 mL of artificial saliva (pH 6.8). Gallic acid was used as standard (linearity range = 5–100 μg/mL; $R^2 > 0.9973$) and the results were expressed as mg of gallic acid equivalents (GAE) per gram of buccal film (mg GAE/g film).

2.8.2. DPPH radical scavenging activity assay

DPPH radical scavenging assay was performed following the procedure described by Barros et al. [39], with minor modifications. Briefly, a film portion with an area of 0.5 cm^2 was dissolved in 1 mL of artificial saliva (pH 6.8). Trolox was used as standard (linearity range = 5–125 μg/mL; $R^2 > 0.9878$). Results were expressed as mg of Trolox equivalents (TE) per gram of buccal film (mg TE/g film).

2.8.3. ABTS radical scavenging activity assay

ABTS radical scavenging assay was performed according to Re et al. [40], with minor modifications. A film portion with an area of 0.5 cm^2 was dissolved in 1 mL of artificial saliva (pH 6.8). Ascorbic acid was used as a standard (linearity range = 5–100 μg/mL; $R^2 > 0.9911$) and the results were expressed as mg of ascorbic acid equivalent (AAE) per gram of buccal film (mg AAE/g film).

2.8.4. Ferric reducing antioxidant power assay

Ferric reducing antioxidant power (FRAP) assay was performed according to Benzie and Strain [41], with minor modifications. A film portion with an area of 0.5 cm^2 was dissolved in 1 mL of artificial saliva (pH 6.8). Ferrous sulphate heptahydrate ($FeSO_4 \cdot 7H_2O$) was used as standard (linearity range = 25–500 μM; $R^2 > 0.9922$). The results were

expressed in μmol of ferrous sulphate equivalents (FSE) per gram of buccal film (μmol FSE/g film).

2.9. In vitro cell viability assays

The biocompatibility of the produced buccal films was assessed by cell viability studies using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. TR146 (human buccal cell line) and HSC-3 (human tongue squamous carcinoma cell line) were grown in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% Fetal Bovine Serum, 1% essential amino acids and 1% antibiotic. The cells were maintained at a temperature of 37 °C with 5% CO_2 and the culture medium changed every two days until reached confluence. In each well of 96-well plates, the cells were seeded at a concentration of 25×10^3 cells/well and incubated for 48 h at 37 °C with 5% CO_2 to provide exponential growth. After incubation, the medium was removed, and the cells were incubated with different films concentrations (77.92–2.44 mg/mL), dissolved in the respective medium, for 24 h. Afterwards, the MTT was added to each well and incubated for 3 h at 37 °C with 5% of CO_2. The optical density was measured at a wavelength of 570 and 630 nm (SynergyTM HT Multi-mode microplate reader, BioTek Instruments Inc.; Winooski, VT, USA). DMEM culture medium was used as positive control and Triton X-100 1% (*w/v*) as negative control. The cell viability (CV) was calculated considering the control group, untreated, as 100% (Eq. (3)):

$$CV(\%) = \frac{A_{570} - A_{630}\ treat\ cell}{A_{570} - A_{630}\ untreat\ cell} \times 100 \quad (3)$$

2.10. In vitro and ex vivo permeation assays

2.10.1. In vitro permeation assay

The advanced buccal epithelium monolayer model was performed according to Teubl et al. [27], with minor modifications. Films were evaluated, along with the *A. arguta* fruit extract. Briefly, TR146 cells were cultured in the apical side of 12-well Transwell® inserts (cellQART®,3 μm pore diameter, polycarbonate, 1.12 cm^2) with a cell seeding density of 2.4×10^4 cells/cm^2, for 30 days. The medium was changed every 2–3 days. At defined timepoints (15, 30, 45, 60, 90, 120, 180 min), 200 μL samples of the basolateral side was collected and the same volume of HBSS was added. Samples were stored at −20 °C and further analyzed by LC/DAD-ESI-MS, as described in section 2.7, in order to assess the content of polyphenols that were able to translocate through the cell layers. The permeability results were expressed as permeation percentage (%), calculated as the ratio between the mass of each compound that permeated the cell layer in the buccal model and the mass of each compound present in the film solution initially applied to the *in vitro* model, and apparent permeability (P_{app}) that was calculated using the following equation (Eq. (4)):

$$P_{app} = \mathrm{dQ} / \mathrm{dt}(\mathrm{A} \times C_0) \quad (4)$$

where dQ is the amount of compound detected in the basolateral side (μg), A is the surface area of the insert (cm^2), C_0 is the initial concentration in the apical compartment (μg/mL), and dt is the time of the experiment(s). The coefficient dQ/dt represents the steady-state flux of each compound across the monolayer.

The transepithelial electrical resistance (TEER) was measured in the advanced cell culture models with an EVOM Epithelial Volthometer Instrument equipped with a chopstick electrode (World Precision Instruments, Sarasota, FL, USA) every 2–3 days (before changing the medium) and were also conducted during the entire permeability assay at each assessed timepoint. Briefly, after 15 min of sterilization, followed by 15 min of stabilization of the electrode tip in cell culture medium or HBSS, the electrode tip was immersed between the apical and basolateral of the Transwell models, avoiding contact with cells, and then resistance (Ω) was measured.

2.10.2. Ex vivo permeation assay

The *ex vivo* permeation assay was carried out in glass Franz diffusion cells (PermeGear, Inc., Hellertown, PA, USA). Briefly, porcine buccal mucosa (total exposed area 0.785 cm^2) was fixed between the donor and the receptor compartments of the Franz cell after being hydrated with phosphate buffer (PBS) (pH 7.4) for 10 min. The receptor chamber was filled with 5 mL of PBS, kept in continuously stirred by a magnetic bar and maintained at 37 ± 1 °C. The samples were prepared by diluting a film portion (4 cm^2) in 1 mL of PBS. Afterwards, 500 μL of sample was added to the donor compartment. At appropriate intervals (0 min, 15 min, 30 min, 45 min, 1 h, 1 h 30, 2 h, 2 h 30, 3 h, 3 h 30, 4 h, 5 h, 6 h, 7 h and 8 h) 300 μL of solution of the receptor chamber were withdrawn with a syringe and immediately replaced with equal volume of fresh PBS. After 8 h, a sample from the donor chamber was collected and the portion of the porcine buccal mucosa used in the assay was transferred to eppendorfs. The samples were stored at - 18 °C until quantification by LC/DAD-ESI-MS, according to the procedure described in section 2.7. The results were expressed as permeation percentage (%), calculated as the ratio between the mass of each compound that permeated the porcine buccal mucosa sample in the Franz diffusion cells, and the mass of each compound present in the film solution initially applied to the *ex vivo* model.

2.11. Statistical analysis

All measurements were performed in triplicate ($n = 3$) and the results were expressed as mean ± standard deviation (SD). A value of $p < 0.05$ was considered significant after the one-way analysis of variance (ANOVA) and the Turkey's HSD test, through the IBM SPSS Statistics 27.0 software (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Physical and chemical properties of the buccal film

The physical and chemical properties of a film can significantly impact the dosage accuracy, the compound release behavior, the handling features, the patient comfort and, most important, its effectiveness. To ensure that buccal films are suitable for their purpose, it is essential to examine their microstructure, weight and thickness consistency, flexibility, ability to endure the movements inside the buccal cavity, as well as their capacity to effectively deliver the bioactive compounds [31]. The hydration ability (moisture content and swelling capacity) and the disintegration time are also crucial factors that need to be evaluated [31]. Table 1 summarizes the obtained results for the buccal films developed.

3.1.1. Mass and thickness uniformity

The films' weight determines the uniform distribution of the active

Table 1
Weight, thickness, moisture content, swelling capacity, and disintegration time of the buccal films.

Sample	Weight (mg)	Thickness (mm)	Moisture content (%)	Swelling capacity (%)	Disintegration time (min)
Control buccal film	131.4 ± 11.7[a]	0.28 ± 0.03[a]	17.46 ± 1.36[a]	63.17 ± 8.28[a]	9.00 ± 1.45[a]
A. arguta fruit extract buccal film	194.8 ± 12.0 [b]	0.37 ± 0.04 [b]	10.53 ± 1.81 [b]	55.95 ± 4.17[a]	15.05 ± 3.25 [b]

Different letters ([a], [b]) in the same column mean significant differences ($p < 0.05$) between samples.

compounds [34], which directly affects its effectiveness and therapeutic impact. Thickness, mechanical qualities, handling features, and compound release behavior are properties greatly influenced by weight, along with flexibility and conformability [31]. According to Table 1, the control buccal films were significantly ($p < 0.05$) lighter (131.4 *vs* 194.8 mg) and thinner (0.28 *vs* 0.37 mm) than the *A. arguta* extract ones. Normally, films for oral application present thickness between 50 and 1000 μm [31], being in line with the obtained results. De Caro et al. developed buccal films with *Aphanizomenon flos-aquae*, a blue-green alga, and reported thicknesses between 0.488 and 0.604 mm, being the *A. arguta* fruit extract buccal films thinner [42]. Concerning mass uniformity, the incorporation of *A. arguta* extract led to a mass increase when compared to the control, despite the films are still lighter. For example, the *Aphanizomenon Flos-Aquae* films presented masses between 232 and 242 mg [42]. Nevertheless, mass greatly depends on the amount of polymer, plasticizer and active compounds incorporated in the formulation, as well as the films dimensions selected to conduct this evaluation [43].

3.1.2. Folding endurance

Folding endurance reflects the mechanical strength and durability of films [33]. None of the buccal films formulated in the present study broken when manually folded (180°), reaching the set limit of 300 folds. Therefore, it is expected that the buccal films produced will be able to withstand the movements of the buccal cavity after administration, being also predictable their processing and packaging without damage.

3.1.3. Hydration ability

Hydration ability should be carefully evaluated since it may greatly impact the film performance, from mechanical properties to the release profile [30,34]. Despite no significant differences ($p > 0.05$) were observed between the control and the *A. arguta* fruit extract buccal films in what concerns to swelling capacity, the control ones presented a significantly higher moisture content (17.46% *vs* 10.53%). In fact, moisture is a crucial parameter of films: in low amounts may result in brittleness or reduced flexibility [43], while in high quantities the water molecules can interpose the polymer chains [34] and lead to sticky films that adhere to patients fingers or packaging, enabling the microbial growth [43]. Considering the significant moisture reduction of the control buccal films (17.46%) when compared to the *A. arguta* fruit extract ones (10.53%), it is hypothesized that the addition of *A. arguta* extract may lead to some matrix discontinuities, facilitating the water loss during the dehydration phase. Nevertheless, the moisture content is adequate since HPMC K100 LV EP, a cellulose derivate, was used as polymer and naturally absorbs water [44].

In what concerns to the swelling capacity, the control buccal films achieved higher results (63.17%) than the *A. arguta* fruit extract ones (55.95%), which was expected given the hydrophilic properties of the polymer used [43,45,46]. This property is extremely important since it greatly influences the films' ability to release the bioactive compounds [31], as well as the patient comfort [42]. The diffusion rate of the bioactive compounds from the *A. arguta* extract incorporated in the buccal films to the oral mucosa will be defined, among other characteristics, by their ability to absorb water [46] and create a gel-like layer, allowing the compounds to be entrapped within the film and preventing it from being released all at once [42]. This capacity is extremely useful in the OM situations since it allow the wound exudate to be absorbed, reducing the bacterial burden, and improving the overall healing [46].

3.1.4. Disintegration time

Considering that the buccal films' purpose is a continuous release of the incorporated potential therapeutic compounds into the buccal cavity, it is necessary to ensure that films do not disintegrate instantaneously. Therefore, the disintegration time was assessed in artificial saliva (pH 6.9), being quantified in 9.00 min for the control buccal films, with significant differences ($p < 0.05$) for the *A. arguta* extract ones (15.05 min). This data is consistent with the swelling capacity determined for both films (63.17% and 55.95%, respectively, for the control and the *A. arguta* fruit extract buccal films). As the buccal films swell upon contact with saliva, it is possible to observe structural modifications that enable their disintegration and the bioactive compounds release [33]. Therefore, a film with higher swelling capacity

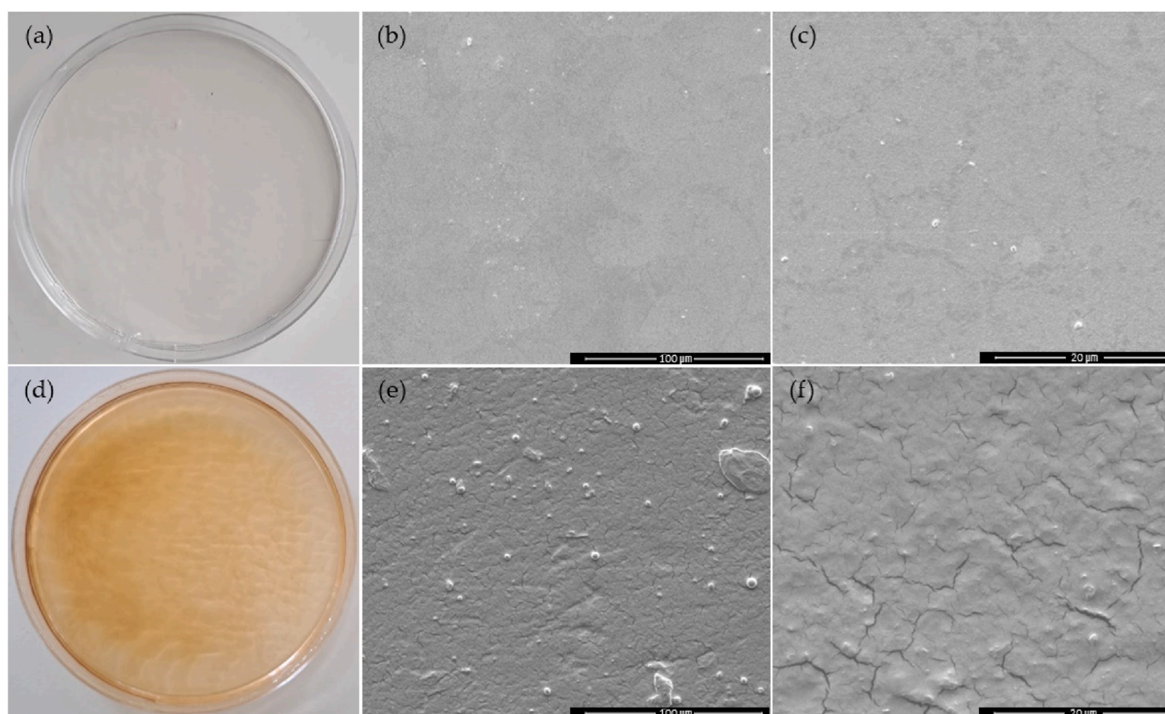

Fig. 1. Images of (a, b and c) control buccal film and (d, e and f) *A. arguta* fruit extract buccal film (a) and (d) are macroscopic images; (b) and (e) are SEM images with a magnification of 1,000×; (c) and (f) are SEM images with a magnification of 5,000×. Beam intensity (HV) of 2,00 kV.

disintegrates faster [47]. Moreover, the highest moisture content observed for the *A. arguta* fruit extract buccal films (17.46%) suggests that the polymeric matrix is more tight, making difficult the water penetration and, thus, leading to a slower disintegration [34]. Also, thinner, and lighter films tend to disintegrate more quickly [30,32,44].

3.1.5. Microstructure analysis

Fig. 1 represents the macroscopic images and the SEM micrographs of the produced films.

In general, it confirms the homogeneous and smooth surface of the formulated films, which is in accordance with previous studies that reported HMPC as one of the few polymers capable of forming intact and functional films [45,48,49]. Regarding macroscopic images (Fig. 1 (a) and (d)), films feature partial transparency and are easily handled. In what concerns to the SEM images, the control buccal films (Fig. 1 (b) and (c)) show a smooth and homogeneous surface, in contrast to the *A. arguta* extract ones (Fig. 1 (e) and (f)) that presented small crystals distributed in a continuous matrix. This may be due to the addition of the *A. arguta* extract that disrupt some of the inter- and intramolecular interactions of the polymer molecules, resulting in a less compact matrix [24]. In this sense, the *A. arguta* fruit extract may not have entirely dissolved in the polymer but instead dispersed in the matrix, resulting in a phase separation between the extract and the HPMC molecules [34]. This phenomenon have been observed in previous studies where different active ingredients, such as caffeine [47], starch [48], tea tree essential oil [49], or corn starch [50], were incorporated in buccal films. Nevertheless, the *A. arguta* fruit extract buccal films presented an acceptable dense and compact morphology, suggesting a great structural integrity and compatibility between the different components.

3.2. Mechanical properties

An ideal buccal film should exhibit adequate flexibility and elasticity, being malleable to prevent breaking from handling and oral activity movements, but not too flexible that extends easily or deforms during the cutting or packaging procedures [31,43]. The integrity and performance of a film can be evaluated by the resistance to extension, the percent of elongation and the Young's Modulus [31,33]. Noteworthy, all these parameters are deeply affected by the buccal films' composition, namely the concentration and type of plasticizer, the active ingredients and the polymer used [24,30]. Table 2 summarizes the mechanical properties of the formulated buccal films.

The resistance to extension, also known as tensile strength, intends to evaluate the maximum force or load that a film can withstand before breaking or undergoing permanent deformation [33], reflecting the film's mechanical strength and durability [29]. According to Table 2, no significant differences ($p = 0.400$) were observed between the control and the *A. arguta* fruit extract buccal films (11.51 N and 10.11 N, respectively). The plasticizer type and its quantity influences the resistance to extension [31]. Therefore, since the control and the *A. arguta* fruit extract buccal films have the same amount of plasticizer, the resistance to extension is similar. Moreover, based on the results obtained, it can be assumed that the addition of *A. arguta* fruit extract did not affect the films tensile strength.

Table 2
Mechanical properties of the formulated buccal films: resistance to extension, percent of elongation and Young's modulus.

Sample	Resistance to extension (N)	Percent of elongation (%)	Young's modulus (MPa)
Control buccal film	11.51 ± 2.04^{a}	109.33 ± 12.9^{a}	1140 ± 290^{a}
A. arguta fruit extract buccal film	10.11 ± 2.32^{a}	36.10 ± 3.94 ^{b}	3440 ± 1020 ^{b}

Different letters (^{a}, ^{b}) in the same column mean significant differences ($p < 0.05$) between samples.

Percent of elongation measures the film deformation before it fails or breaks [29], indicating the flexibility and the ability of the film to conform to the oral cavity contours or to withstand deformation without fracturing. It has been reported that the polymer type and content, the amount of plasticizer and the bioactive compounds have a profound effect on the film percent of elongation [31]. The *A. arguta* fruit extract buccal films exhibited a significantly lower ($p = 0.001$) percent of elongation than the control buccal films (36.10% *vs* 109.33%), which is in line with other studies where the addition of the active ingredient (*Panax ginseng* extract [30], starch [48], tea tree essential oil [49], and corn starch [50]) lead to a decrease of the percent of elongation when compared to the controls. This probably occurs due to the interaction of the extract compounds with the polymer through hydroxyl groups, preventing the polymer chain from sliding during the mechanical evaluations [51].

Finally, the Young's modulus, also known as elastic modulus or stiffness [31], represents the resistance of a film to deformation under applied stress and measures the film ability to return to its original shape after the stress removal [29,33]. The control buccal films displayed a significantly ($p = 0.002$) higher Young's Modulus (3440 MPa) than the *A. arguta* fruit extract buccal films (1140 MPa), indicating that the extract buccal film is stiffer, less deformable, and more resistant to bending or stretching. Different physical films features may influence this mechanical property [30]. For example, Young's modulus tends to increase when a film is heavier or thicker. A thicker and heavier film may have a denser polymer matrix and is, therefore, richer in inter- and intramolecular interactions [52], leading to stiffness films and higher Young's modulus. Hydration ability can also play a crucial role in films elasticity, since the mobility of the polymer chains may be influenced by the presence of water molecules in the polymeric matrix [43,45]. Among the formulated films, the *A. arguta* fruit extract buccal films had the lowest moisture content (10.53% *vs* 17.46%) and swelling capacity (55.95% *vs* 63.17%), which may indicate the lower deformation capacity reflected by the higher Young's modulus obtained (3440 MPa *vs*

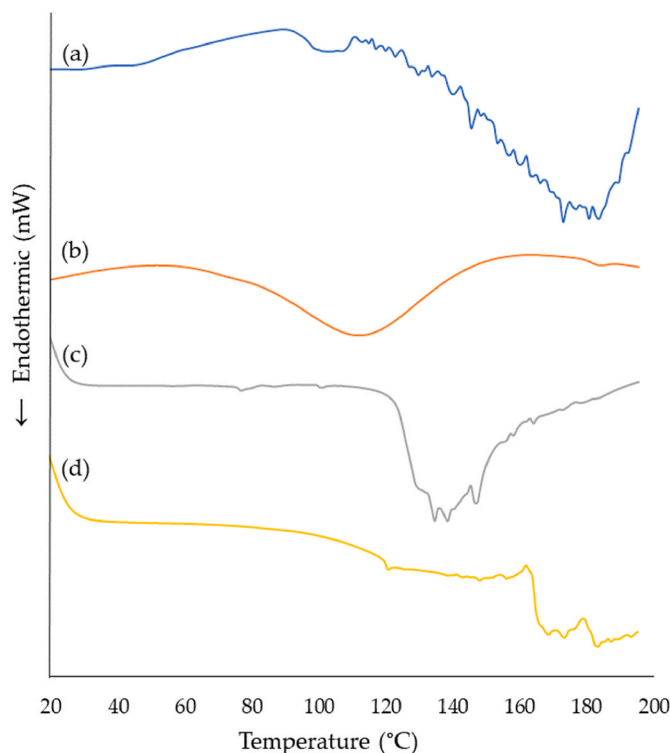

Fig. 2. DSC thermograms of (a) *A. arguta* fruit extract, (b) the polymer used to formulate the films (HPMC K100 LV EP), and both films produced, (c) control buccal film and (d) *A. arguta* fruit extract buccal film.

1140 MPa). Still, the results obtained are in line with the results reported by other authors [30,48–50].

Given the mechanical properties evaluated, it can be affirmed that the *A. arguta* fruit extract buccal films are more resistant than the control ones, ensuring their resistance to handling, transportation, and administration, minimizing the risk of damage or failure. Moreover, the *A. arguta* fruit extract buccal films may also resist to stress or tension during application or removal from packaging.

3.3. Differential Scanning Calorimetry (DSC)

The thermal properties and stability of molecules inside films can be determined by DSC analysis. The DSC profile of the *A. arguta* fruit extract as well as the formulated films and the polymer used are represented in Fig. 2.

A. arguta fruit extract (Fig. 2 (a)) displayed a progressive endothermic behavior over time, reaching a peak around 180 °C that may represent the melting point of the phytochemical compounds present. According to literature [32,48], HPMC K100 LV EP (Fig. 3 (b)) may withstand to high temperatures and be extruded between 120 and 190 °C, but thermally degrades when heated between 200 and 250 °C. In the present study these temperatures were not employed and, therefore, no exothermic peaks were identified in the polymer thermogram. The slight endothermic peak observed around 100 °C may signify the evaporation of some water present in the sample [53]. Regarding the formulated films, the control buccal films (Fig. 2 (c)) displayed a different thermal profile when compared to the polymer. This film is composed by polymer and plasticizer, therefore the endothermic peak observed between 130 and 160 °C is due to the plasticizer. Regarding the *A. arguta* fruit extract buccal films (Fig. 2 (d)), the characteristic melting peak of the extract was absent, suggesting that it transitioned from a crystalline (hard) to an amorphous state (brittle) during the solvent casting process. Although this form presented a reduced stability, it can improve the compounds solubility, leading to an increased bioavailability [32].

Table 3
Total phenolic content (TPC) and antioxidant/antiradical activity (ABTS and DPPH radicals scavenging capacity and ferric reducing antioxidant power (FRAP) assay, respectively) of the formulated buccal films.

Sample	TPC (mg GAE/g film)	FRAP (μmol FSE/g film)	ABTS (mg AAE/g film)	DPPH (mg TE/g film)
Control buccal film	NA	NA	NA	NA
A. arguta fruit extract buccal film	6.46 ± 0.54	49.45 ± 2.41	3.74 ± 0.09	4.90 ± 0.54

GAE, Gallic acid equivalents; FSE, Ferrous sulphate equivalents; AAE, Ascorbic acid equivalents; TE, Trolox equivalents.
NA: No activity was determined up to the highest tested concentration (24.35 mg/mL).

3.4. Biological activity

Table 3 summarizes the antioxidant and antiradical activities of the buccal films evaluated by TPC, FRAP, ABTS and DPPH assays.

As expected, no results were obtained for the control buccal films. The *A. arguta* fruit extract buccal films displayed a TPC of 6.46 mg GAE/g film and considerable antioxidant/antiradical activities (FRAP = 49.45 μmol FSE/g film; ABTS = 3.74 mg AAE/g film and DPPH = 4.90 mg TE/g film). Recently, our research team [19] performed these assays with the *A. arguta* fruit extract used in this study and achieved a TPC of 18.71 mg GAE/g dw and a FRAP result of 186.88 μmol FSE/g dw, while the ABTS scavenging capacity was 16.33 mg AAE/g dw. Since the films and the extract results are expressed in different units, it is not possible to compare them. Yet, the films' results are expected to be lower than the extracts' ones since the polymer or the plasticizer may act as interferents in the assays performed. For example, the TPC assay is based on the reduction of the Folin–Ciocalteu reagent by phenolic compounds [38]. Therefore, any compound present in the film that binds to the Folin–Ciocalteu reagent or prevents phenolic compounds from binding to it may be an interferent. Given that one of the roles of the polymer is precisely to prevent the release of phenolic compounds immediately

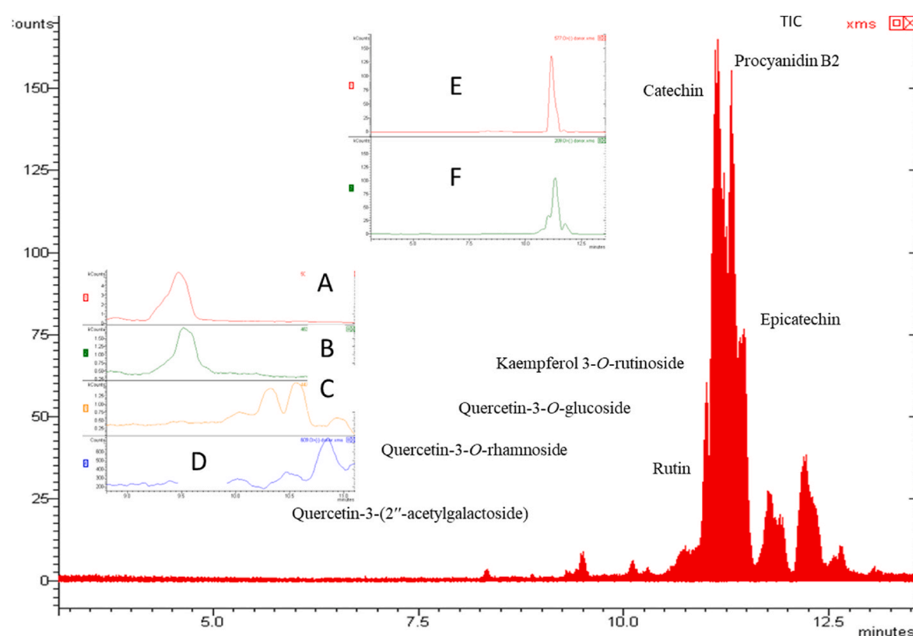

Fig. 3. LC-ESI-MS chromatogram of the *A. arguta* fruit extract buccal film; the chromatogram is represented by the Total Ion Current (TIC), main peaks related to identified compounds are highlighted. Chromatograms corresponding to the m/z values used for each peak are shown and letters are indicating mrm traces for quercetin-3-(2″-acetylgalactoside) (A), quercetin-3-*O*-rhamnoside (B), quercetin-3-*O*-glucoside (C), rutin (D), catechin and epicatechin (E), procyanidin B2 (F). The identified peaks are also indicated in Table 4.

Table 4
Identification and quantification of the phytochemical compounds present in the *A. arguta* fruit extract buccal film through LC/DAD-ESI-MS analysis.

Peak	Compound	Rt (min)	[M − H]⁻ (*m/z*)	Quantification (mg/100 g film) *
	Flavonol			
1	Quercetin-3-(2″-acetylgalactoside)	9.45	505	37.24 ± 1.86
2	Quercetin-3-*O*-rhamnoside	9.50	463	143.79 ± 7.19
3	Quercetin-3-*O*-glucoside	10.5	447	6.34 ± 0.32
5	Kaempferol 3-*O*-rutinoside (isomer 1)	11.0	593	17.49 ± 0.92
8	Rutin	11.6	609	14.95 ± 0.75
9	Kaempferol 3-*O*-rutinoside (isomer 2)	11.9	593	29.35 ± 1.47
	∑ Flavonol			**250.16 ± 10.00**
	Flavan-3-ol			
4	Catechin	11.0	289	202.25 ± 10.11
6	Procyanidin B2	11.1	577	1108.83 ± 55.44
7	Epicatechin	11.4	289	929.16 ± 46.46
	∑ Flavan-3-ol			**2240.24 ± 89.61**

Rt – Retention time.

after administration, causing an entrapment effect by forming complexes with the compounds [51], the polymer represents a potential interference. This phenomenon has been reported in previous studies, namely by Baranauskaite et al. [30] that produced a film containing *Panax ginseng* extract with TPC and antioxidant/antiradical activities lower than the respective extract.

3.5. Phytochemical composition

To establish the potential of the buccal films to prevent or even treat the OM symptoms, it is essential to assess the presence of natural bioactive compounds with relevant biological activity in their composition. Therefore, the films' phytochemical composition was analyzed by LC/DAD-ESI-MS. Fig. 3 presents the chromatograms obtained for the *A. arguta* fruit extract buccal films, while Table 4 summarizes the different compounds identified based on the retention time and the fragment ions detected by the mass spectrometer, along with the qualification of each one.

As can be observed in Table 4, a total of nine flavonoids were identified and quantified in the *A. arguta* fruit extract buccal films: six flavonols (2240.24 mg/100 g film) and three flavan-3-ol (250.16 mg/100 g film). Among these categories, flanan-3-ol (peaks 4, 6 and 7) were the compounds quantified in greater amounts, with procyanidin B2 (1108.83 mg/100 g film) being the major one, followed by epicatechin (929.16 mg/100 g film) and catechin (202.25 mg/100 g film). Regarding flanovols (peaks 1, 2, 3, 5, 8 e 9), quercetin-3-*O*-rhamnoside (143.79 mg/100 g film) was the compound identified and quantified in higher amounts in *A. arguta* fruit extract buccal films, followed by quercetin-3-(2″-acetylgalactoside) (37.24 mg/100 g film) and kaempferol 3-*O*-rutinoside (isomer 2) (29.35 mg/100 g film). It is possible to conclude that the incorporation of the *A. arguta* fruit extract in the buccal films was successfully achieved, as significant amounts of several compounds previously identified in the extract by our team, such as catechin, epicatechin, and quercetin [19], were detected in the buccal films.

Considering that the buccal films developed are intended to be applied in the prevention/treatment of OM, and given the multistep nature of the OM development process [5,7,54], the natural bioactive compounds identified in the *A. arguta* fruit extract buccal films may act in different phases. The ionizing radiation usually used in radiotherapy treatments disrupt the delicate balance within the oral mucosa, leading to the generation of ROS that in turn triggers a cascade of events [4,8], including oxidative stress, inflammation, and cellular damage. The pivotal role of ROS in OM pathogenesis make them a target for therapeutic strategies [7], particularly natural bioactive compounds with well reported antioxidant properties, capable of scavenging free radicals and enhance endogenous antioxidant defenses (e.g. enzymes) [4]. For example, rutin, procyanidin B2, epicatechin, quercetin and respective derivatives are well known for their powerful free-radical scavenger capacity and modulation of ROS production pathways, for example by inhibiting LDL oxidative damage and lipid peroxidation process [54,55]. Their effectiveness to prevent and treat OM have already been demonstrated by multiple *in vitro* and *in vivo* assays [6,56]. Moreover, the anti-inflammatory properties of kaempferol-3-*O*-rutinoside, catechin and quercetin derivatives [54,56,57] are extremely useful in mitigating the OM development [7]. An additional aspect that must be considered when trying to treat or prevent OM is the microorganisms action [8]. One of the basic characteristics of OM is ulceration that comprises the integrity of the oral cavity [4], allowing the action of microorganisms and increasing, drastically, the risk of sepsis [5,54]. Therefore, the presence of compounds such as catechin and kaempferol-3-*O*-rutinoside, well known antimicrobials [58], is an added advantage in the buccal films developed.

3.6. In vitro cell studies

Fig. 4 summarizes the viability of HSC-3 and TR146 cell lines after exposure to the formulated buccal films assessed by an MTT assay. These cell lines were selected as buccal models to evaluate the potential effects on the oral cavity, since HSC-3 is a human tongue squamous carcinoma cell line, while TR146 is a keratinized cell line derived from the human squamous buccal carcinoma [19].

As can be observe in Fig. 4, the buccal films did not affect the viability of both cell lines in none of the concentrations tested, presenting viabilities between 93% and 114%. In what concerns to the control buccal films, the TR146 cells viability was around 100%, with similar results for the HSC-3 cell line, after 48 h of exposure to concentrations between 2.44 and 77.92 mg/mL, with no significant differences ($p = 0.188$ and 0.599, respectively). Regarding *A. arguta* fruit extract buccal films, the viability of TR146 cells was 111.49 %, while for HSC-3 this value was 102.49% after exposure to the highest concentration (77.92 mg/mL), without significant differences ($p = 0.792$ and 0.720, respectively). Our research team previously assessed the influence of the *A. arguta* fruit extract in the same cell lines [19], in concentrations between 125 and 2000 μg/mL. As reported, the extract did not decrease the TR146 viability, except for the concentration of 500 μg/mL that led to a viability of 86.20%. In what concerns to the HSC-3 cell line, it was possible to observe a viability decrease (between 69.14% and 56.33%) for the highest concentrations tested (500 μg/mL and 2000 μg/mL, respectively). Considering the results achieved and the oral cavity structure, these results emphasize the non-cytotoxic effect of the produced buccal films.

3.7. Permeation studies

3.7.1. In vitro permeation assay

One of the main challenges for the market introduction of buccal formulations is the difficulty in accurately assessing the permeability through the oral mucosa [28,59]. Although *in vivo* permeation assays are the most precise, due to cost and ethical issues the *in vitro* permeability models are commonly used, being fundamental steps to predicting bioavailability and delivery of natural compounds [59]. In the present study the safety and bioavailability of the developed buccal films were evaluated in an *in vitro* buccal model composed by TR146 cells [27]. Fig. 5 presents the *in vitro A. arguta* fruit extract buccal film release profile considering the different polyphenols identified and quantified after the *in vitro* model buccal permeation assay at the different time points, as well as Supplementary Table 1. Table 5 and Table 6 display the apparent permeability coefficient and the steady-state flux of the

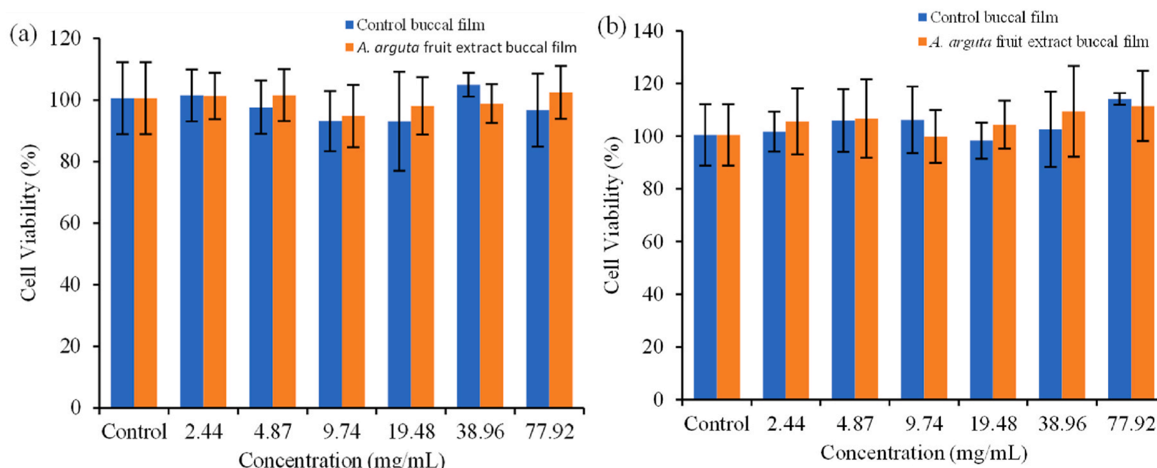

Fig. 4. Effect of the formulated buccal films on the viability of (a) HSC-3 and (b) TR146 cell lines measured by an MTT assay ($n = 3$), at different concentrations (77.92–2.44 mg/mL).

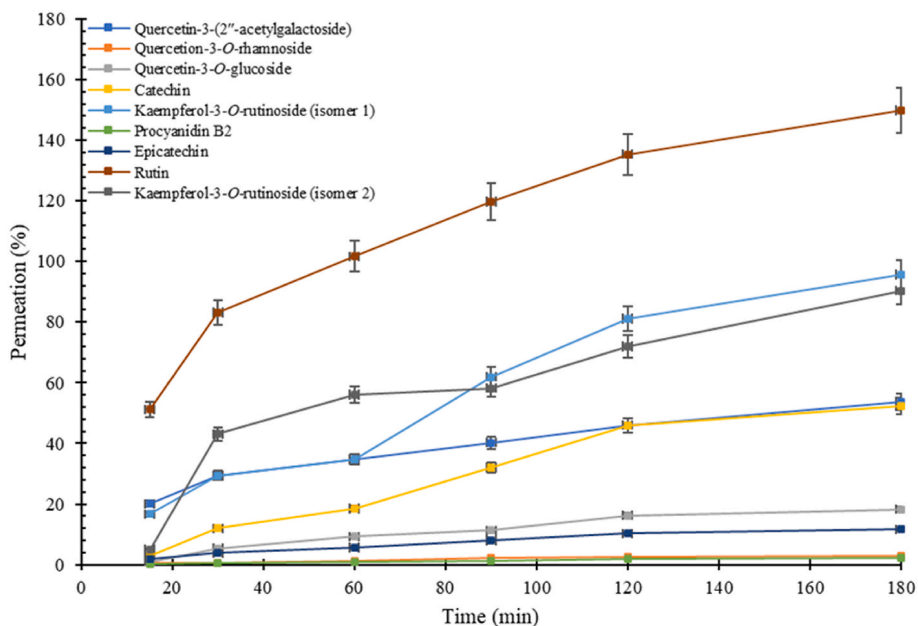

Fig. 5. Permeation profile of the compounds present in the *A. arguta* fruit extract buccal film capable of permeating the cell layer in the *in vitro* permeation assay at different timepoint.

compounds found in the *A. arguta* fruit extract buccal film during the *in vitro* permeation assay at various time points.

Rutin (Rt: 11.6 min; peak 8) was the compound detected in higher amounts in most of the timepoints, reaching a permeability of 149.90% at the final timepoint (180 min), followed by kaempferol 3-*O*-rutinoside (isomer 1) (Rt: 11.0 min; peak 5; 95.64%) and kaempferol 3-*O*-rutinoside (isomer 2) (Rt: 11.9 min; peak 9; 90.33%). Overall, procyanidin B2 (Rt: 11.1 min; peak 6) was the compound detected in lower amounts in all timepoints, with a permeability that varied between 0.04% (15 min) and 2.29% (180 min).

Multiple parameters may influence the compounds' buccal permeation, such as concentration, molecular size and structure, hydrophilicity, permeation time, and TEER [26,44,60,61]. Hydrophilic compounds with larger molecular sizes tend to struggle to cross cell membranes, which can be the case of procyanidin B2 (2.29%), a catechin dimer with a large and complex structure. In addition, it is possible that buccal cells, such as TR146, have used these compounds' antioxidant and anti-inflammatory properties for their own maintenance, similar to intestinal cells (Caco-2 and HT29-MTX cell lines) [55]. Regarding catechin and epicatechin, although both compounds are hydrophilic and shown a small molecular size, the different permeation percentages obtained (55.28% and 11.60%, respectively) may be due to differences in molecular structures, since they are stereoisomers of each other [54]. In what concerns to the quercetin glycosylated derivatives, the different permeation may be attributed to the glycoside moiety. This type of structural modification can influence the compounds' properties, such as increase molecular size and hydrophilicity [54]. For instance, the galactose acetylation in quercetin-3-(2″-acetylgalactoside) (53.65%) may have increase the molecular size of the compound, but also promoted beneficial interactions with hydrophobic and hydrophilic regions of the cellular environment, leading to a higher permeation. Nevertheless, considering the oral cavity environment, several metabolic reactions may take place and disrupt glycosidic bonds [59], preventing the presence of glycosylated compounds to be detected in the permeates. This may be the reason for quercetin-3-*O*-rhamnoside (2.95%), and quercetin-3-*O*-glucoside (18.29%) being detected in lower percentages.

Table 5
Apparent permeability coefficient (P_{app} x 10^{-6} (cm/s)) of the compounds present in the *A. arguta* fruit extract buccal film in the *in vitro* permeation assay at different timepoint.

P_{app} x 10^{-6} (cm/s)	Time (min)					
	15	30	60	90	120	180
Quercetin-3-(2″-acetylgalactoside)	79.6 ± 3.98	58.4 ± 2.92	34.5 ± 1.73	26.7 ± 1.34	22.8 ± 1.14	17.7 ± 0.89
Quercetin-3-*O*-rhamnoside	2.7 ± 0.14	1.4 ± 0.07	1.2 ± 0.06	1.4 ± 0.07	1.2 ± 0.06	1.00 ± 0.05
Quercetin-3-*O*-glucoside	3.5 ± 0.17	10.7 ± 0.53	9.1 ± 0.46	7.5 ± 0.37	8.0 ± 0.40	6.1 ± 0.30
Catechin	11.9 ± 0.60	23.6 ± 0.18	18.4 ± 0.92	21.2 ± 1.06	22.8 ± 1.14	17.3 ± 0.87
Kaempferol 3-*O*-rutinoside (isomer 1)	66.4 ± 3.32	58.2 ± 2.91	34.5 ± 1.73	41.0 ± 2.05	40.3 ± 2.02	31.6 ± 1.58
Procyanidin B2	0.2 ± 0.008	0.8 ± 0.042	1.00 ± 0.049	0.8 ± 0.039	0.9 ± 0.047	0.8 ± 0.040
Epicatechin	7.9 ± 0.40	7.5 ± 1.18	5.6 ± 0.92	3.2 ± 1.06	5.1 ± 1.14	3.8 ± 0.87
Rutin	203.0 ± 10.1	165.0 ± 8.3	101.0 ± 2.8	79.2 ± 1.9	67.2 ± 1.8	49.6 ± 1.5
Kaempferol 3-*O*-rutinoside (isomer 2)	20.1 ± 1.0	85.6 ± 4.3	55.6 ± 2.8	38.5 ± 1.9	35.7 ± 1.8	29.9 ± 1.5

Table 6
Steady-state flux of the compounds present in the *A. arguta* fruit extract buccal film in the *in vitro* permeation assay at different timepoint.

Steady-state flux (μ/s)	Time (min)					
	15	30	60	90	120	180
Quercetin-3-(2″-acetylgalactoside)	0.13 ± 0.007	0.10 ± 0.005	0.06 ± 0.003	0.04 ± 0.002	0.04 ± 0.002	0.03 ± 0.001
Quercetin-3-*O*-rhamnoside	0.05 ± 0.003	0.03 ± 0.001	0.02 ± 0.001	0.03 ± 0.001	0.02 ± 0.001	0.02 ± 0.001
Quercetin-3-*O*-glucoside	0.03 ± 0.001	0.09 ± 0.004	0.07 ± 0.004	0.06 ± 0.003	0.07 ± 0.003	0.05 ± 0.002
Catechin	0.63 ± 0.031	1.25 ± 0.062	0.97 ± 0.048	1.12 ± 0.056	1.20 ± 0.060	0.91 ± 0.046
Kaempferol 3-*O*-rutinoside (isomer 1)	0.11 ± 0.006	0.10 ± 0.005	0.06 ± 0.003	0.07 ± 0.004	0.07 ± 0.003	0.05 ± 0.003
Procyanidin B2	0.03 ± 0.001	0.17 ± 0.008	0.20 ± 0.010	0.16 ± 0.008	0.19 ± 0.009	0.15 ± 0.008
Epicatechin	2.41 ± 0.120	2.29 ± 0.115	1.69 ± 0.085	1.61 ± 0.081	1.55 ± 0.077	1.17 ± 0.058
Rutin	0.22 ± 0.011	0.18 ± 0.009	0.11 ± 0.006	0.09 ± 0.004	0.07 ± 0.004	0.05 ± 0.003
Kaempferol 3-*O*-rutinoside (isomer 2)	0.04 ± 0.002	0.17 ± 0.009	0.11 ± 0.006	0.08 ± 0.004	0.07 ± 0.004	0.06 ± 0.003

Regarding rutin, also known as quercetin-3-*O*-rutinoside (149.90%), despite being the largest compound from the quercetin derivatives, the presence of multiple rings and hydroxyl groups (polar structure) might contribute to the increased polarity of this compound, as the overall structure may allow for optimal interactions with cellular membranes, taking advantage of paracellular transporters [59]. Furthermore, being a quercetin glycoside, rutin could theoretically result from the metabolic degradation of other quercetin derivatives [26], such as quercetin-3-*O*-rhamnoside or quercetin-3-*O*-glucoside, which justify the permeation observed. Concerning kaempferol-3-*O*-rutinoside isomers 1 (95.64%) and 2 (90.33%), the hydrophilic profile of the compounds, coupled to its capacity to interact with different components of the cell membrane, may have conducted to high permeation percentages. These preliminary results allow to verify that the compounds present in the *A. arguta* fruit extract buccal films can permeate the buccal mucosa. However, it is noteworthy to mention that this permeation study was performed using a static model, not accounting with swallowing, salivation, and masticatory movements, aspects that may favor the permeation of some compounds or, on the contrary, promote their degradation/structural alteration. Considering the positive *in vitro* results achieved, an *ex vivo* assay was performed with porcine mucosa (section 3.7.2).

Throughout this study TEER, a non-invasive method used to monitor living cells during various stages of growth and differentiation [60], was measured for 31 days to guarantee the integrity and permeability of the model, as well as during the permeability assay to ensure the viability of the process [60]. As observed in Fig. 6 (a), the TEER values remained stable for the seeding days (175 ± 35 Ω/cm^2). On the experiment day, during the permeability assay, the TEER values ranged between 161 Ω/cm^2 and 201 Ω/cm^2 (Fig. 6 (b)).

This data attest that the application of the *A. arguta* fruit extract buccal films did not affect the tight junctions function in the TR146 cell layer and, therefore, the compounds present in the formulated films are most likely delivered via a transcellular route [59].

3.7.2. *Ex vivo permeation assay*

Freshly excised mucosa is widely used as barrier membrane in diffusion studies to closely resemble the *in vivo* permeation scenario, given the similarity in terms of histological organization and lipid composition to human mucosa [29]. Porcine buccal mucosa appears to be the most suitable animal model considering availability, thickness, and permeation properties, as attested by different authors [31,33]. In the present study, porcine buccal mucosa was used along with Franz diffusion cells to evaluate the permeation of the *A. arguta* extract compounds incorporated in the films produced. Fig. 7 represents the release profile of the phytochemical compounds present in the *A. arguta* fruit extract buccal films that permeated the porcine buccal mucosa in the *ex vivo* permeation assay at the different time points, as well as Supplementary Table 2.

Quercetin-3-*O*-glucoside (Rt: 10.5 min; peak 3) was the compound detected in higher amounts in most of the timepoints (between 10.12% and 84.95%), although at the final time point (480 min), quercetin-3-(2″-acetylgalactoside) (Rt: 9.45 min; peak 1) showed a higher permeation (112.26%). Overall, kaempferol 3-*O*-rutinoside (isomer 2) (Rt: 11.9 min; peak 9) was the compound identified in lower amounts in all timepoints, reaching a permeability that varied between 1.15% (15 min) and 14.77% (480 min).

Multiple parameters are responsible for the compounds' interactions with the porcine buccal mucosa, impacting their ability to permeate through the tissue barrier placed in the Franz diffusion cell, such as size, lipophilicity, glycosidic moieties, and structural complexity [62]. Catechin and epicatechin are relatively small monomers, which justify the high permeation observed (79.74 and 47.05, respectively). The slightly lower permeability of epicatechin may be due to subtle structural differences, such as the presence of two chiral carbons (positions 2 and 3) that lead to diastereomers [54]. As expected, since procyanidin B2 (21.23%) is a dimer of catechin [55], the larger size and the more complex structure may hinder its permeation through the oral mucosa, contributing to a lower permeability. Additionally, buccal cells may employ this compound for self-maintenance. Given the antioxidant and anti-inflammatory properties of flavan-3-ols, the oral cavity may experience the same situation, as intestinal cells (Caco-2 and HT29-MTX) have already demonstrated [55]. Regarding the glycosylated flavonols,

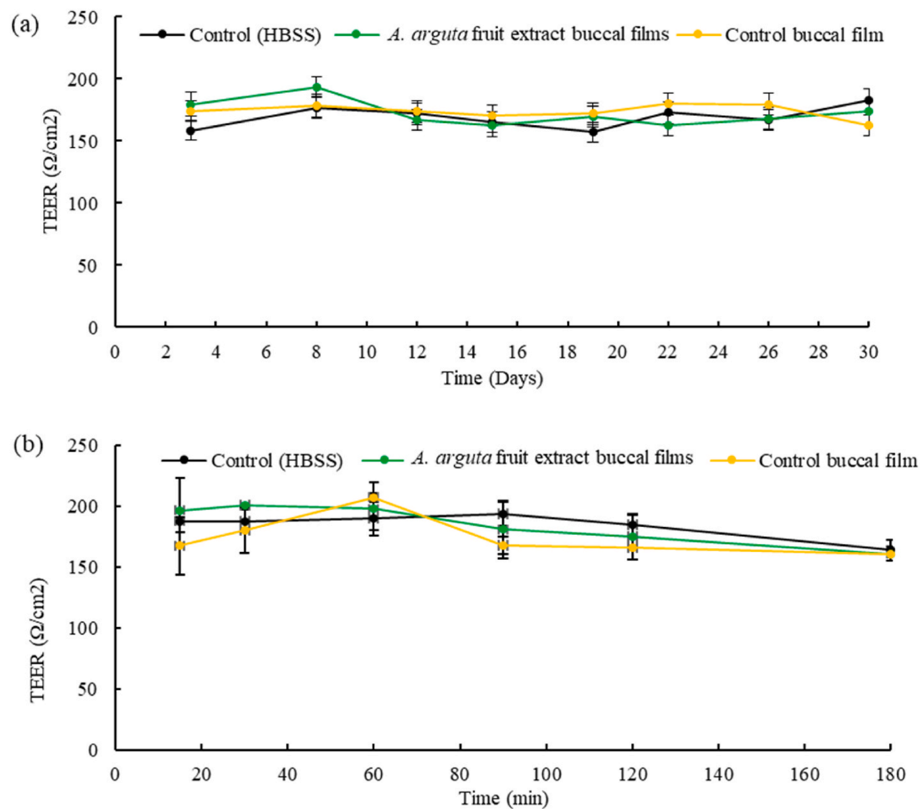

Fig. 6. TEER measurements of the 3D buccal model (TR146) monitored (a) during 31 days in Transwell™ membranes and (b) during the 180 min of the permeability assay.

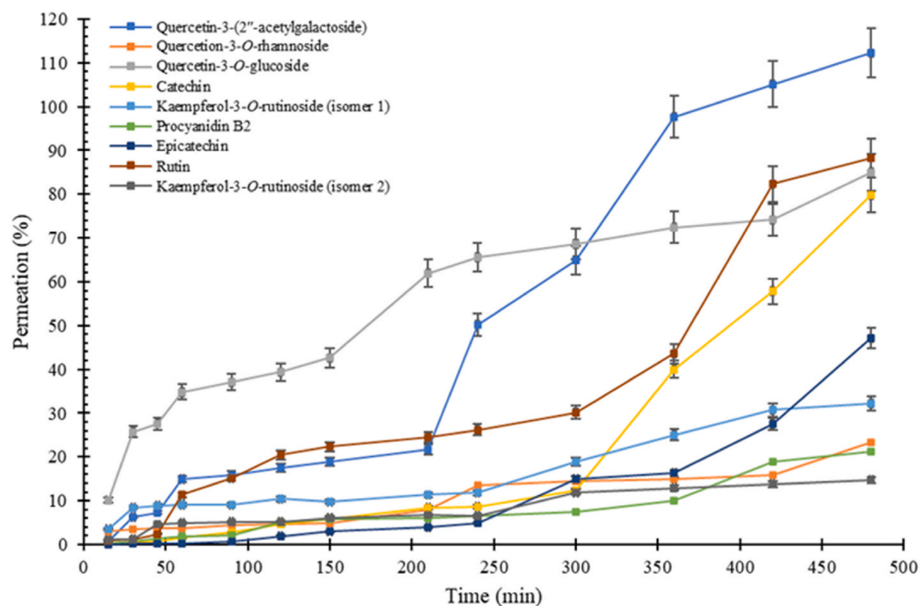

Fig. 7. Permeation profile of the compounds present in the *A. arguta* fruit extract buccal film capable of permeating the porcine buccal mucosa at different timepoints.

the different permeation may be attributed to the glycoside moiety. The small structure of glucose may allow quercetin-3-*O*-glucoside to present an efficient permeation (84.95%), in contrast to quercetin-3-*O*--rhamnoside (23.41%), since rhamnose is a larger glycoside moiety. Despite the high molecular size of rutin (88.32%), the chemical structure with multiple rings and hydroxyl groups (polar structure) create a favorable environment for interactions with the mucous membrane, allowing the permeation by alternative mechanisms, such as transporters [59]. In what respects to kaempferol-3-*O*-rutinoside, both isomer 1 (32.21%) and 2 (14.77%) result from the glycosylation and hydroxyl protection of the 3-OH group of kaempferol [57]. These unique isomeric structural arrangements may impact the overall molecular configuration, explaining the permeation differences. The same may occur to quercetin-3-(2″-acetylgalactoside) (112.26%), since the

acetylgalactoside fraction might enhance permeability, potentially due to improved lipophilicity and interactions with the buccal mucosa [54].

It should be also highlighted that some compounds may undergo structural modifications or be metabolized in the oral cavity given its structure and activity (presence of saliva and its components or external factors provided by food consumption and other daily activities) [59], and either not being detected in the permeates, or resulting in different compounds. This may justify the permeability percentage above 100% of quercetin-3-(2″-acetylgalactoside). It is possible that some of the flavonoids identified in the *A. arguta* fruit extract buccal films, under favorable environmental conditions and in the presence of relevant enzymes, are metabolized into other compounds and identified in the permeates as being another compound different from the one initially identified in the buccal films.

Overall, relevant permeations were obtained for all the compounds identified in the *A. arguta* fruit extract buccal films, which is extremely positive as it reaffirms the usefulness of the film to prevent OM. The different permeations reported for the same compounds through the *in vitro* and *ex vivo* assays are essentially due to the fact that tissues are multi-layered when compared to cells [28]. Nevertheless, it is possible to affirm that the compounds present in the *A. arguta* fruit extract buccal films are capable of permeating both models and exert their local effects. Future *in vivo* studies in animal models will be crucial to support the efficacy demonstrated by the *in vitro* and *ex vivo* assays.

4. Conclusions

In the present study, the formulation of buccal films with *A. arguta* fruit extract was successfully achieved. The buccal films developed were easy to handle and presented adequate physical properties, such as weight, thickness, folding endurance, disintegration time, moisture content and swelling capacity. The mechanical features were also assessed, and the flexibility and resistance to stress were proven. The results revealed that the compounds from *A. arguta* fruit extract may have acted as cross-linking agents, turning the *A. arguta* fruit extract buccal films less water soluble, more rigid, less stretchable, and more opaque than the control ones. Most importantly, the *A. arguta* fruit extract buccal films achieved excellent results for the antioxidant/antiradical activities, highlighting the role of procyanidin B2, epicatechin, and quercetin-3-*O*-rhamnoside as predominant compounds. Regarding the buccal cells' viability, it was not affected for both films formulated, which reinforced its safety. Moreover, the *in vitro* and *ex vivo* permeation studies performed attested that the compounds present in the *A. arguta* fruit extract buccal films are capable of permeating both cells and multilayered tissues, with rutin, quercetin-3-*O*-glucoside and catechin being the principal ones. Overall, this work showed the potential and safety of buccal films containing *A. arguta* fruit extract for OM prevention. Further studies should be carried out, including the analysis of the impact of long-term storage, the anti-inflammatory and antimicrobial properties of the films and, most important, *in vivo* assays in animals to ensure the compounds bioavailability.

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CRediT authorship contribution statement

Filipa Teixeira: Writing – original draft, Software, Methodology, Investigation, Formal analysis. **Ana Margarida Silva:** Methodology, Investigation, Formal analysis. **Catarina Macedo:** Methodology, Investigation, Formal analysis. **Berta Estevinho:** Methodology, Investigation, Formal analysis. **Stefania Sut:** Methodology, Investigation, Formal analysis. **Stefano Dall'Acqua:** Methodology, Investigation, Formal analysis. **Cristina Delerue-Matos:** Resources, Methodology. **Paulo C. Costa:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization. **Francisca Rodrigues:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jddst.2024.105725.

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