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Production and Validation of *Opuntia ficus-indica* derived cosmeceuticals

Abstract

Currently, in the cosmetics market has been a crescent demand for ingredients with bioactive effects such as antioxidants, anti-ageing and UV protectors, preferably from non-synthetic sources, such as plant extracts.

Opuntia ficus-indica is a native species from Mexico existing widespread in almost all regions of the world, including Portugal. Belonging to Cactaceae family possess high adaptability to habit arid climes. Traditionally it is associated as a source of nutrition providing fruits, cladodes and flowers for human consumption. Nevertheless, a high amount of cladodes are used as forage or disposed in landfills. Furthermore, the pharmacological properties of cladodes have been studied due to their antidiabetic, wound healing, and anti-inflammatory properties. Acknowledging their pharmacological potentialities, an *Opuntia ficus-indica* cladodes hydroalcoholic extract was evaluated on the phenolic content, *in vitro* antoxidant activity, antimicrobial activity and cytotoxic effect of in order to develop a cosmeceutical.

HPLC-DAD analysis of cladodes hydroalcoholic extract revealed a high content of glycosides derivates of quercetin. The cladodes extract showed an antioxidant activity with a significant reduction of radical ABTS (EC_{50} of 0.23 $mg.ml^{-1}$). The cytotoxic effect of cladodes extract was evaluated in fibroblast cell line L929. This extract did not show cytotoxicity in the concentration range of 0.05 to 1 $mg.ml^{-1}$. Further ahead, a cosmeceutical containing cladodes extract was developed. This cosmeceutical was submitted to *in vivo* evaluation on the skin of volunteers with the non-invasive probes Corneometer®, Tewameter® and Mexameter®, which measures the hydration, trans-epidermal water loss and erythema, respectively. The cosmeceutical demonstrated a moisturizing effect with the increasing of hydration values for 3 hours and 6 hours, and not showed an allergenic effect.

Keywords: antioxidant, cladodes, cosmeceutical, extract, phenolics

Produção e Validação de cosmecêuticos derivados de *Opuntia ficus-inidica*

Resumo

Atualmente, no mercado dos cosméticos tem havido uma exigência crescente por ingredientes com efeitos bioativos, como antioxidantes, antienvelhecimento e protetores UV, preferencialmente de fontes não sintéticas, como extratos vegetais.

Opuntia ficus-indica é uma espécie nativa do México existente em quase todas as regiões do mundo, incluindo Portugal. Pertence à família Cactaceae possuindo uma alta adaptabilidade para habitar climas áridos. Tradicionalmente é utilizada como fonte de nutrição, consumindo-se frutos, cladódios e flores. No entanto, uma grande quantidade de cladódios é usada como forragem ou descartada em aterros sanitários. As propriedades farmacológicas dos cladódios tem sido alvo de estudo, baseando-se nas suas propriedades antidiabéticas, cicatrizantes e anti-inflamatórias. Reconhecendo estas potencialidades farmacológicas, neste trabalho, foram avaliados: o conteúdo em compostos fenólicos, a atividade antioxidante *in vitro*, a atividade antimicrobiana e o efeito citotóxico de um extrato hidroalcoólico de cladódios com o objetivo de desenvolver um cosmecêutico.

A análise por HPLC-DAD do extrato hidroalcoólico de cladódios revelou um teor elevado de derivados glicosídeos da quercetina. O extrato de cladódios mostrou atividade antioxidante com uma redução significativa do radical ABTS (EC_{50} de 0.23 mg.ml^{-1}). Foi avaliado o efeito citotóxico do extrato de cladódios na linha celular de fibroblastos L929. Este extrato não mostrou citotoxicidade nas concentrações testadas entre 0.05 a 1 mg.ml^{-1}. Posteriormente foi desenvolvido um cosmecêutico com extrato de cladódios incorporado. Este cosmecêutico foi submetido a uma avaliação *in vivo* na pele de voluntários com as sondas não invasivas: Corneometer®, Tewameter® e Mexameter® que medem a hidratação, a perda de água trans-epidérmica e o eritema, respetivamente. O cosmecêutico demonstrou efeito hidratante com o aumento dos valores de hidratação durante 3 horas e 6 horas, e não mostrou efeito alergénico.

Palavras-chave: antioxidante, cladódios, cosmecêutico, extrato, fenólicos

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List of abbreviations and acronyms

$^{1}O_{2}$	Oxygen Singlet
AA	Ascorbic acid
ABTS	2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)
BC	Base cream
CAM	Crassulacean acid metabolism
CAT	Catalase
CC	Cladode cream
CE	Cladodes extract
CFU	Colony forming unity
COX-2	Cyclooxygenase-2
DMSO	Dimethyl sulfoxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
Dwr	Dry weight residual
EC_{50}	Half maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
FRAP	Ferric ion reducing antioxidant power
Fwb	Fresh weight biomass
Fz	Ferrozine
GPX	Glutathione peroxidase
GR	Glutathione reductase
HO·	Hydroxyl radical
HPLC	High performance liquid chromatography
HPLC-DAD	High performance liquid chromatography diode array
ICA	Iron chelating activity
IL-6	Interkulin-6
iNOS	Nitric oxide synthase
LOO·	Lipid peroxyl radical
LPS	Lipopolysaccharide
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
Na$_2$EDTA	Ethylenediaminetetraacetic acid disodium salt
NADH	Nicotinamide adenine dinucleotide
NBT	Nitroblue tetrazolium
NED	N-(1-naphthyl) ethylenediamine
NO	Oxide Nitric
NO·	Oxide nitric radical
O$_2$	Oxygen
O$_2^-$·	Superoxide anion
OD	Optical density
ONOO$^-$	Peroxynitrite
ONOOH	Peroxynitrous acid
PBS	Phosphate buffer saline
PMS	Phenazine methosulfate
ROS	Reactive oxygen species
SNP	Sodium nitroprusside
SO	Superoxide
SOD	Superoxide Dismutase
T	Trolox
TCC	2,3,5-Triphenyltetrazolium chloride
TNF-α	Tumor necrosis factor alpha
TWEL	Transepidermal water loss
U	Untreated
UV	Ultraviolet
UVA	Ultraviolet A-rays
UVB	Ultraviolet B-rays
UV-Vis	Ultraviolet-Visible

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1.Introduction

1. Introduction

1.1. *Opuntia ficus-indica*

Opuntia ficus-indica is worldwide known as prickly pear and in Portugal as "figueira da Índia" or "piteira". The genus *Opuntia* belong to Cactaceae family (Andreu et al. 2018), which are plants with CAM metabolism (Taiz and Zeiger, 2002). This type of metabolism makes the plants capable of habit in water-scarce areas and arid regions because of efficient capacity to retain water by opening the stomas at night for CO_2 capture (Taiz and Zeiger, 2002). Originated from Mexico is distributed throughout South and Central America, North and South Africa, Mediterranean regions, India and Australia (Aragona et al., 2018; El-Mostafa et al., 2014; FAO, 2013). It is believed that worldwide spread of *O. ficus-indica* was by European travels in XV century, due to a special interest in a red and purple dye, produced from the *Dactylopius coccus* insect who colonize the cactus. This dye became very valuable between XV to XIX century (Griffith, 2004).

Recently, *O. ficus-indica* cultivars represent an economical source for many countries like Mexico, Italy, Morocco, Peru, South Africa and Tunisia, being Mexico, the biggest worldwide producer of the species followed by Italy (FAO, 2013). Sicily retains more than 90 % of *O. ficus-indica* production in Italy (FAO, 2013; Ginestra et al., 2009; Rocchetti et al., 2018), representing an important economical and food source for the country (Rocchetti et al., 2018).

The *O. ficus-indica* fruits varieties, in general, are *Gialla* (yellow fruit), *Rossa* (red fruit) and *Bianca* (white fruit) (Aragona et al., 2018). This variety of fruit colours is due to the combinations of two betalains pigments that are betanin (purple-red) and indicaxanthin (yellow-orange) (Aragona et al., 2018; Butera et al., 2002). Usually, the fruit is consumed fresh or transformed in different products like juices, jams, alcohol, sweeteners and dehydrated sheets (Saenz et al., 2002). The juice from the fruits has antiulcerogenic and hepatoprotection proprieties that are attributed to the flavonoid and mineral content (Galati et al., 2003; Galati et al., 2005). Flowers colours range from

pink, purple, to white and yellow, being yellow the predominant (Aragona et al., 2018). Flowers are poorly studied because of the short floriation period, but diuretic proprieties from decoctions and infusions are described (Galati et al., 2002; Aragona et al., 2018).

Cladodes are widely used for forage or fertilizers (Rocchetti et al., 2018). Besides, cladodes are transformed into several products like pickles, marmalades, jams, candy, flour, sauce and alcoholic beverages (Saenz et al., 2002). Usually, red fruits are used to produce a traditional know alcohol beverage, namely "colonche". However, the cladodes along with fruits are also used for alcoholic fermentation were 60 % of cladodes sugar is converted in alcohol (Saenz et al., 2002). Cladodes are also a source of bioenergy in Chile and Mexico, used for biogas production with an energetic efficiency of 364 000 Mcal/hect-year with a volume of 52 000 m^3/hect-year (Crimina et al., 2018). Despite these usual usages, the cladodes possess several pharmacological potentialities that could be applied to add value products like dietary supplements or cosmeceuticals.

1.2. *Opuntia ficus-indica* in Portugal

O. ficus-indica is wildly distributed in Portugal, commonly found in the road borders and gardens. It began to gain commercial value a few years ago, with market demands for transformed products or fresh fruit consume, seed oil and powdered cladodes (Alves and Oliveira, 2014; Clara, 2015). Cultivation began in Algarve, where is located the largest plantation, followed by other productive areas distributed in Castelo Branco, Évora, Portalegre, Beja, Santarém, Leiria, Bragança, Viseu, Setúbal and Guarda (Clara, 2015). This geographic amplitude can be attributed to optimal climacteric conditions provide by Portugal geographic localization (Clara 2015). *Rossa* variety is cultivated in larger proportions due to the special interest of national and international markets (Alves and Oliveira, 2014).

The company *Cactacea* at Sesimbra was the first in Portugal to have *O. ficus-indica* cultivated in an orderly orchard with 0,8 hectare in 2009 (Clara, 2015). Cactacea also commercializes transformed *O. ficus-indica* like tea flower, liquors, and jams (Alves and Oliveira, 2014). Nevertheless, *O. ficus-indica* products are still poorly known in the national market (Alves and Oliveira, 2014).

1.3. Chemical characterization of cladodes

Water is the main component of fresh cladodes (80-95%). The main constituents in whole dry cladodes are polysaccharides (96 %) of mucilage, lignin, cellulose, hemicellulose and pectin (Rocchetti et al., 2018; Malainine et al., 2003). The mucilage is a complex hydrocolloid composed by more than 30,000 monosaccharides, where the majority is associated with uranic acids (Bayar et al., 2016; Felkai-Haddache et al., 2016). This hydrocolloid is capable of retaining water and regulate calcium fluxes (Bayar et al., 2016; Ginestra et al.,2009). The pectin is also a polysaccharide, which is mainly composed of galacturonic acids with neutrals sugars in the side-chains (Felkai-Haddache et al., 2016). These two polymers are interesting for food industries due to their gelling properties, used as a food thickener, food emulsifier, water purifier and adhesive lime (Bayar et al., 2016; Felkai-Haddache et al., 2016).

The monosaccharides content of cladodes are rhamnose (7.71 μg.mg^{-1}), fucose (4.42 μg.mg^{-1}), arabinose (39.64 μg.mg^{-1}), xylose (18.64 μg.mg^{-1}) mannose (13.64 μg.mg^{-1}), galactose (33.69 μg.mg^{-1}), glucose (153.15 μg.mg^{-1}) and galacturonic acid (96.26 μg.mg^{-1}) (Ginestra et al., 2009).

Cladodes are also rich in putative bioactive compounds like phenolics. The most abundant phenolics compounds found are derivates of cyanidin (428.27 mg.kg^{-1}), pelargonidin (161.39 mg.kg^{-1}), petunidin (114.08 mg.kg^{-1}) and phenolic acids like hydroxybenzoics (114.01 mg.kg^{-1}), hydroxyphenylopropanoics (91.58 mg.kg^{-1}) and hydroxycinnamics (1248.24 mg.kg^{-1}) (Rocchetti et al.,

2018). Ginestra and collaborators (2009) also identified phenolic compounds like glucosyl forms of isorhamnetin and piscidic acid which is the main simple phenolic found.

1.4. Cladodes bioactivities

Several investigators have studied cladodes bioactivities using *in vitro* and *in vivo* models, these bioactivities are indicated in table 1.

Cladodes have been used for skin health promotion, like wound healing and UV (ultraviolet) protection. The wound healing activity was evaluated by *in vitro* cellular model based on a scratched keratinocytes monolayer by Lorenzo and collaborators (2017), who demonstrated that cell migration and proliferation in "wound closure" was enhanced by the cladodes mucilage. In addition, the topical application of cladodes methanolic extracts in rats showed wound healing properties by measuring the strength of skin strips from the wound segments (Park and Chun, 2001). The UV protective activity of cladodes water extracts was evaluated using keratinocytes cells irradiated with UVA, the expected cleavage of caspase-3 and caspase-7 induced by UV radiation was interrupt by cladodes extract, preventing apoptosis (Petruk et al., 2017).

The inflammation is a response of the immune system to defend our organism against harmful agents (Chen et al., 2018). Furthermore, prolonged acute inflammation could induce additional tissue damaged (Chen et al., 2018). The cladodes extract and the isorhamnetin glycosides isolated from cladodes showed anti-inflammatory properties by inhibition of COX-2 and the reduction in levels of NO, IL-6, and TNF-α *in vitro* and *in vivo* assays (Antunes-Ricardo et al., 2015).

Diabetes is the most worldwide commonly disease in developing countries as a result of an unhealthy lifestyle (Atlas, 2015). Cladodes revealed anti-hyperglycemic and hypoglycemic effects due to the flour viscosity, property that reduce the glucose levels by entrapment mechanisms and delay of intestinal absorption (Nuñez-López et al., 2013). Another study with diabetic rats showed that

cladode water extract significantly corrects disconcerted carbohydrate metabolism (Hwang et al., 2017). In addition, a study with humans showed that cladode intake in a high carbohydrate breakfast promotes anti-hyperglycemic and anti-hyperinsulinemic effects and in high soy protein breakfast interrupted postprandial blood glucose peaks (López-Romero et al., 2014).

Infusion of cladodes demonstrated anti-obesity effect by reducing triglycerides levels, plasma total cholesterol and plasma glucose in mice treated with high-fat diet and dextran sulphate sodium (induced ulcerative colitis) (Aboura et al., 2017). In addition, cladodes infusion inhibited colon shortening, the infiltration of macrophage cells and suppressed mucosal injury in colon tissues (Aboura et al., 2017). Cladode intake decreased hepatic steatosis by increasing fatty acid oxidation, very-low-density lipoproteins synthesis, decreasing oxidative stress and improving liver insulin signalling in non-alcoholic fatty liver disease mice (Morán-Ramos et al., 2012).

Cladodes provide a diet rich in calcium that in mice showed to be bioavailable and enhance mineral density, physical, mechanical and microstructural properties of femur bones (Hernández-Becerra et al., 2017). An *in vitro* study of cladodes extract showed protective effects against oxidative damage in chondrocytes cells (Panico et al., 2017). In addition, the intake of cladodes as a source of calcium on diet showed an improvement of bone mineral density in a woman with low bone density (Aguilera-Barreira et al., 2013).

The oral intake of gel and aqueous extract of cladodes showed significant diuretic effects in mice (Bakour et al., 2017). Moreover, the lyophilized cladode extract administered intravenously, in rabbit, also showed diuretic effects (Bakour et al., 2017).

The aqueous extracts of cladodes demonstrated nephron-protective effect against sodium dichromate-induced kidney damage in mice (Mbarka et al., 2019) and showed antimicrobial activity against *Porphyromonas gingivalis* and *Prevotella intermedia* bacteria from periodontitis patients (Arbia et al., 2017). In addition, cladodes methanolic extract demonstrated anti-spasmodic activity

by relaxing the spontaneous and high K+ (80 mM)-induced contraction in rabbit jejunum (Lanuzza et al., 2017).

Table 1. Cladode biological properties.

Bioactivities properties	References
Against non-alcoholic fatty liver disease	Morán-Ramos et al., 2012
Antibacterial	Arbia et al., 2017
Anti-inflammatory	Antunes-Ricardo et al., 2015
Anti-obesity	Aboura et al., 2017;
Anti-osteoporosis, Anti-articular diseases	Aguilera-Barreira et al., 2013; Hernández-Becerra et al., 2017; Panico et al., 2007
Anti-spasmodic	Lanuzza et al., 2017
Diuretic	Bakour et al., 2017
Hypoglycemic	Hwang et al., 2017; Nuñez-López et al., 2013;
Nephroprotective	Mbarka et al., 2019
UV-Protective	Petruk et al., 2017
Wound healing	Lorenzo et al., 2017; Park and Chun, 2001

1.5. ROS (reactive oxygen species) and antioxidants defences on skin

Human skin is the largest organ on human body, covering an area about 1.7 m^2 and representing 15 % of the total body weight (Zaind and Lanigan, 2010), is composed by the epidermidis, the dermis and the hypodermis (figure 1) (Zaind and Lanigan, 2010). The skin acts as defence barrier against environmental hazards that might induce oxidative damage.
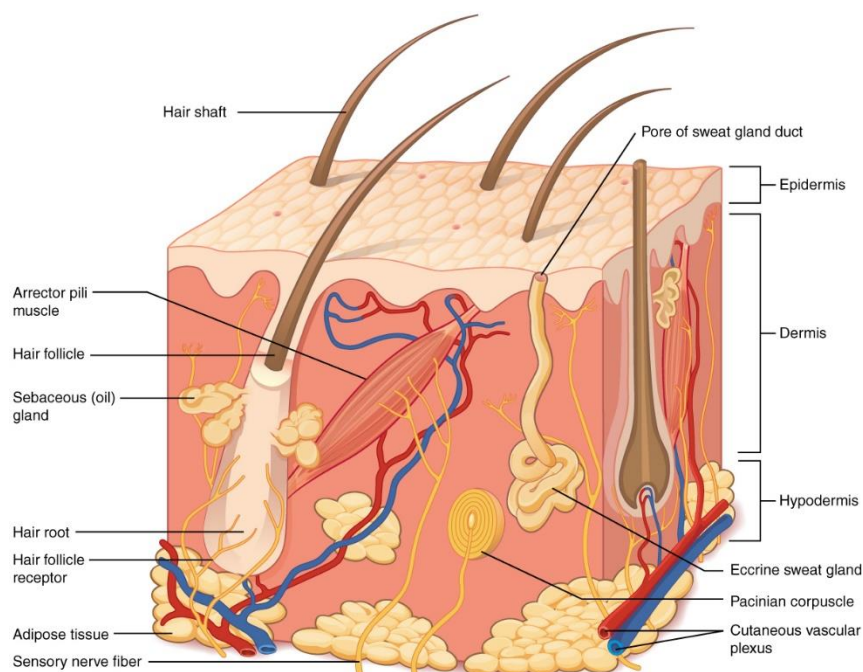
Figure 1. Skin structure by Donna Browne, Integumentary System Module 2: Layers of the Skin. OpenStax CNX.

The skin is expose to several harmful agents from environmental that can cause oxidative damage, for example the UV radiation and the air pollutants induce ROS generation like superoxide anion radical (O_2^{-}), the hydroxyl radical ($HO^{\cdot}$), lipid peroxyl radical ($LOO^{\cdot}$) and nitric oxide radicals ($NO^{\cdot}$) (Masaki, 2010; Kim et al., 2016).

The human skin has enzymatic mechanisms to maintain ROS homeostasis, like superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and catalase (CAT) (Masaki, 2010; Pinell and Durham, 2003). For example, the SOD catalyses the $O_2^{-\cdot}$ converting into a more stable molecule like the hydrogen peroxide (H_2O_2) and the oxygen molecule (O_2) (Masaki, 2010). The hydroxyl radical (OH) is the more unstable radical and it might damage lipids, proteins and DNA (Schieber and Chandel, 2014). Besides, the hydrogen peroxide can react with Cu^{+} and Fe^{2+} to forms hydroxyls radicals via Fenton reaction (Halliwell, 1997; Matés et al.,1999) or via the Haber-Weiss reaction between $O_2^{-}\cdot$ and H_2O_2 (Matés et al.,1999).

The oxide nitric free radical (NO) is endogenously synthesized by inflammatory responses. However, the excess of NO production can be cytotoxic. Its reaction with $O_2^-\cdot$ result in $ONOO^-$ formation which reacts with hydrogen to form ONOOH and is decomposed in OH (Freeman, 1994; Rubbo et al., 1996).

The UV radiation is the major cause of skin aging displaying wrinkles, dryness, telangiectasia, and pigmentary abnormalities (Pinell et al., 2003). The UVB radiation is completely absorbed in the epidermidis and the UVA radiation is capable to reach the dermis (Pinell et al., 2003). The UVB mostly induced $O_2^-\cdot$ generation through activation of nicotinamide adenine dinucleotide phosphate oxidase (NADPH) and the respiratory chain reactions (Masaki, 2010). The UVA interacts with photosensitizers or chromophores such as riboflavin and porphyrin which lead to the production of singlet oxygen ($^1O_2$) (Musaki, 2010; Chen et al., 2012). Consequently, ROS accumulation could generate a cascade of reactions that leads to lipid peroxidation comprising the cellular integrity, DNA damage like modifying the guanine nucleotide (8-hydroxyguanine) leading to carcinogenesis and proteins oxidation (Chen et al., 2012). The UV exposure also induces an inflammatory response, especially the erythema from sunburn, triggered by $NO\cdot$ through prostaglandin E2 synthesis (Masaki, 2010). Furthermore, the UV radiation induces skin pigmentation through $NO\cdot$ release by the keratinocytes (cells of epidermis) increasing the melanogenic factors tyrosinase and tyrosinase-related protein 1 (Masaki, 2010). The particulate matter from the air pollutants like the smog, cigarette smoke and burned of organic matter increase the skin aging (Kim et al., 2016). Studies with mice that were exposed to O_3 showed stratum corneum (the more external layer of epidermidis) damage, exhibiting a significant level of malondialdehyde (MDA) (parameter of lipid peroxidation) (Thiele et al., 1997; Thiele, 2001). In addition, it was demonstrated that air pollutants induced skin tumour by up-regulating oxidative stress, pro-inflammatory cytokines, and cell proliferation (Kim et al., 2016). For example, cigarette smoke extract induces ROS that consequently damage the DNA of

fibroblast leading to skin carcinogenesis (Kim et al., 2016). The two environmental agents previous mentioned, can act synergistically by increasing squamous cell carcinomas (Kim et al., 2016).

Despite the human skin possesses enzymatic defences, daily intake of plants increases antioxidant protection. For example epicatechin, epicatechin-3-gallate, epigallocatechin, and epigallocatechin-3-gallate showed anti-inflammatory and anti-carcinogenic effects on skin (Chen et al., 2012; Pinell and Durham, 2003; Svobodová et al, 2003), resveratrol had antiaging effects and protects the skin cells from ROS induced by UV (Masaki, 2010). The resveratrol and silymarin significantly inhibited skin edema and apigenin inhibited the skin tumour incidence (Svobodová et al., 2003). Intake of ascorbic acid and tocopherol through diet showed to be important for the skin protection, for example ascorbic acid neutralizes the ROS present in aqueous compartment of skin, reduce dark pigmentation and protect the epidermal barrier of the skin (Chen et al., 2012). Whereas, tocopherol protects the stratum corneum layer from lipid peroxidation and protein oxidation (Pinell and Durham, 2003). In addition, ascorbic acid is capable of regenerate the oxidized tocopherol, being a relevant interaction for skin protection (Chen et al., 2012; Pinell and Durham, 2003).

The topical application of antioxidants showed to be important to reduce the ROS generated from the UVA radiation (Chen et al., 2012), presenting direct advantage compared with oral intake supplements, due to, direct action in the skin area that demand protection (Pinell and Durham, 2003). Topical application of (-) epigallocatechin-3-gallate can inhibit the skin tumour formation induced by UV radiation unlike the oral intake (Pinell and Durham, 2003; Gensler et al., 1996).

1.6. Cosmeceuticals nowadays

The term cosmeceuticals refer to cosmetics with bioactive ingredients that provide pharmaceutical benefits to skin. The term was attributed in 1984 crossing the mid-way of "cosme"tics and pharma"ceutical" (Pandey and Sonthalia, 2019). However, this term is not

recognized for the United States Food and Drug Administration or the European Union. The United States consider the cosmeceuticals as a subclass of drugs and the Europe of Union as a subclass of cosmetics (Pandey and Sonthalia, 2019).

According to the Cosmeceutical Market Size & Share: Global Industry Report, in 2018 the global cosmeceutical market size was valued in 49.5 billion dollars. The skincare products category dominated the market with a share of 43.3 % in 2018. The ages between 30 and 40 represent the group that showed the highest demand for skincare cosmetic products. This stage of age is crucial for skin; the years of oxidative damage accumulated evidence the wrinkles. Furthermore, demand for skin cosmetics with medicinal benefits is rising, the consumers are conscious of antioxidants, organic ingredients, and peptides benefits on the skin.

Some preservatives present in cosmetics could induce skin allergenic reactions but also interfere with the endocrine system being possible involved in the development of mammary tumours (Halla et al., 2018). The propylparaben and butylparaben are an example of those preservatives that were recommended at low concentrations by the Scientific Committee on Consumer Safety (SCCS) (Halla et al., 2018). In addition, some "parfum" or "fragrances" contain phthalates that besides of endocrine-disrupting, lead to dysfunctions on cognitive mental and behavioural functions in pre-natal and infancy stages (Nicolopoulou-Stamati et al., 2015). Other substances that induce endocrine disruption are triclosan, perfluorinated, bisphenol, nanoparticles of metal oxides (titanium dioxide and zinc oxide) and the organic UV filter like oxybenzone (Nicolopoulou-Stamati et al., 2015).

Nowadays, the consumers are demanding products composed by plant-based "natural" compounds that are sustainable and microbiome "friendly" (Hilton, 2019). Besides, the constraints previously mentioned enhancing the consumers demand for cosmeceuticals with more "natural" ingredients. The plants extracts could be an alternative for decreasing the use of harmful ingredients,

highlighting the antioxidant capacity of compounds that plant extracts provide also can act as preservatives and UV protectors.

1.7. Objectives

There is evidence that cladodes from *O. ficus-indica* have pharmacological potentiality and the research for the development of products like pharmacological drugs, dietary supplement or cosmeceutical could be promising. Our main objective is the development of a cosmeceutical with the incorporation of an extract of cladodes. For the development of a cosmeceutical was signed the following procedures and proprieties studies of cladodes:

- Cladodes hydroalcoholic extraction;

- Phytochemical analyse of cladode extract using a high-performance liquid chromatography (HPLC);

- Evaluation of *in vitro* antioxidant activity;

- Evaluation of antimicrobial activity;

- Evaluation of cytotoxicity in fibroblast cell line l929 from rats;

- Formulation of a cosmeceutical with incorporation of cladode extract;

- Evaluation *in vivo* of a cosmeceutical containing the cladode extract performed in volunteers using non-invasive probes to measure the skin condition.

2. Material and Methods

2. Material and Methods

2.1. Preparation of *Opuntia ficus-indica* extracts

The extracts of *Opuntia ficus-indica* were prepared from grounded lyophilized cladodes. Briefely, 1 g of cladodes powder was mixed with 10 ml of ethanol-water solution (8:2), extracts were treated with ultrasound for 15 min (Brandson 2510) and stored in the dark at room temperature during seven days. The extract was concentrated in Rotavapor (Buchi RE-121) at a temperature of $40\pm1°C$, frozen at -80°C and lyophilized for 72 hours; residues were resuspended in dimethyl sulfoxide (DMSO, SIGMA, France) and stored at -20°C until further analysis.

2.2. High Performance Liquid Chromatography Diode Array (HPLC-DAD) analysis

2.2.1. Identification of phenolic content

For HPLC analysis, 1 ml from the previous non-evaporated extract (100 mg/ml dw) was filtered using a nylon membrane filter (GelmanSciences, 0.45µm, Michigan, EUA) and stored in the dark at 4°C until further analysis. Also, were prepared standard compounds of caffeic acid, chicory acid, chlorogenic acid, ellagic acid, gallic acid, apigenin, hesperidin, epicatechin, lutenin-7-glucoside, naringenin, quercetin and quercetin-3-glucoside (SIGMA) in order to determine the phenolic compounds content of cladodes extract.

The samples were run in HPLC-DAD (HITACHI, LabChrom Elite, Japan) monitored by the computer software EZChrome elite. The compound separation was performed on a reversed phase LiChroCART column (LiChrospher 100. RP-18e. 5µm, Merk, Germany) at room temperature. The mobile phase used as solvent A, methanol and B, ultrapure water containing formic acid 0.1%. The elution gradient it is described in table 2. The detection was processed in a 250-600 nm range, recording the chromatograms at 260, 280 and 350 nm.

Table 2. Elution specifications for HPLC-DAD analysis

Time (min)	Solvent A %	Solvent B %
0	5	95
5	5	95
30	50	50
40	90	10
45	90	10
50	5	95
60	5	95

2.2.2. Quantification of total phenolic content

Briefly, 1 ml of the extract used for phenolic identification was used for the quantification of total phenolic content by HPLC-DAD analysis. Furthermore, to express the phenolic content in quercetin equivalents were prepared samples with the ranged concentrations of 0.05 - 0.4 5 mg/ml to obtain a calibration curve.

The samples were run in the same HPLC-DAD apparatus and the compound separation was performed on a reversed phase LichroCart 125-4 column (LiChrospher 100-RP-18 (5μm), Merk, Germany). The elution grade was the one showed at table 2, using different solvents: solvent A (acetonitrile containing 0.1 % formic acid) and solvent B (water containing 0.1 % formic acid). The result of quantification total phenolic content was expressed as milligrams of quercetin/ g of dry sample.

2.3.   *In vitro* antioxidant assays

2.3.1. DPPH radical scavenging activity (DPPH)

The 2,2-Diphenyl-1-picrylhydrazyl DPPH assay is widely used, being a simple and rapid method to evaluate the antiradical (antioxidant) potential of a sample (Alam et al., 2013). The DPPH radical in contact with substances that can donate an electron or hydrogen becomes in a reduced

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molecule and the violet colour from the DPPH radical solution decrease (Huang et al., 2005). A DPPH (SIGMA, Spain) 400 µM solution in ethanol was prepared and absorbance was adjusted between 0.9 to 1 in a microplate reader (SpectraMax Plus 384) at 515nm.

The assay was performed as follows: a dilution series of CE at different concentrations (0.1-5 mg.ml⁻¹) were prepared in ethanol. Briefly, 10 µl of CE were mixed with 140 µl of DPPH 400 µM. The 96 well plate was incubated protected from light for 60 minutes. Trolox was used to perform a standard curve calibration and as reference compound for compare extracts activity. DPPH scavenging capacity of extract was calculated using the following equation:

$$\% \, DPPH \, radical \, scavenging \, activity \, = \frac{Abscontrol - (Abssample - Absblank)}{Abscontrol}$$

Abs control- absorbance of work solution

Abs sample- absorbance of CE and work solution

Abs blank- absorbance of CE

2.3.1. ABTS⁻ radical scavenging activity (ABTS)

The free radical ABTS⁻ (2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)) don't exist in human body (Huang et al., 2005), is generated by ABTS²⁻ oxidation when react with potassium persulfate (Alam et al., 2013). To generate the ABTS radical, 88 µl of potassium persulfate at 148 mM was mixed with 5 mL of ABTS (Honeywell Fluka™,Germany) at 27 mM and keep it protected from light at room temperature for 12 to 16 hours. After radical generation, 0.5 ml of previous ABTS solution were added to 44 ml of sodium acetate buffer (20 mM at pH 4.5), the absorbance was adjusted to 0.7 at 750 nm (working solution). The solutions are prepared daily.

The assay was performed as follows: a dilution series of CE at different concentrations (0.05-1.5 mg.ml⁻¹) were prepared in sodium acetate buffer. Briefly, 12 µl of CE were mixed with 188 µl of work solution. The 96 well plate was incubated and protected from the light for 6 min. Trolox (Fisher

Scientific™, USA) was used to perform a standard curve calibration and as reference compound for compare extracts activity. All measurements were performed in triplicate.

The scavenging capacity of extract was calculated with the follow equation:

$$\% \, ABTS \, radical \, scavenging \, activity \, = \frac{Abscontrol - (Abssample - Absblank)}{Abscontrol}$$

Abs control- absorbance of work solution

Abs sample- absorbance of CE and work solution

Abs blank- absorbance of CE

2.3.2. Iron Chelating Activity of Fe^{2+} (ICA)

The iron chelating activity (ICA) assay evaluate the extract capacity of chelating Fe^{2+}. In this procedure, it is used Iron II choride-4-hydrate (FeSO4 ·7H_2O,SIGMA, Spain) and Ferrozine (Fz) (3-(2-pyridyl)-5,6.diphenyl-1,2,4-triazine-4´,4"-disulphonic acid sodium salt, SIGMA, Spain) which react forming Fz-Fe^{2+} coloured complex (Alam et al., 2013). Cladodes extracts (CE) were assessed based on their abality to interfere with the Fz-Fe^{2+} complex.

The assay was perfomed as follows: a dilution series of CE at different concentration (0.1- 5 mg.ml^{-1}) were prepared in ultrapure water. Briefly, 50 µl of CE were mixed with 50 µl of $FeSO_4$·7H_2O stock solution (0.12 mM in ultrapure water) and 50 µl of Fz stock solution (0.6 mM in ultrapure water). After 10 min of incubation in the dark, at room temperature, the absorbance was measured at 570 nm using a microplate reader (Bio-Rad iMark). Na_2EDTA (disodium salt of ethylenediaminetetraacetic acid, SIGMA, USA) was used to perform a standard curve calibration and as reference compund for compare extracts activity. The different solutions were prepared daily and kept protected from the light. All measurements were performed in triplicate.

The iron chelating activity were expressed in percentage using the following equation:

$$\% \text{ Iron chelating activity} = \frac{Abscontrol - (Abssample - Absblank)}{Abscontrol} \times 100$$

Abs control-absorbance of Fz reaction with iron

Abs sample-absorbance of Fz reaction with iron and CE

Abs blank- absorbance of CE without Fz

2.3.3. Ferric ion reducing antioxidant power (FRAP)

The FRAP assay consists in the reduction of [Fe (III) 2,4,6-tripyridyls-triazine (TPTZ)$_2$]$^{3+}$ to [Fe (II) (TPTZ)$_2$]$^{2+}$ (Honeywell Fluka™,Germany). To the reduction capacity assay a TPTZ 10 mM solution in 40 mM of hydrochloric acid (SIGMA), 23 mM of sodium acetate buffer at pH 3.6 and 20 mM of iron (III) chloride hexahydrate (FeCl$_3$.6H$_2$O) in ultrapure water were prepared. For each assay, a work solution was prepared daily, composed by 25 ml of sodium acetate buffer, 2.5 ml of TPTZ and 2.5 ml of FeCl$_3$.6H$_2$O which was previous heated at 37°C. The assay was performed as follows: dilution series of CE at different concentrations (1.5 - 0.05 mg.ml^{-1}) were prepared in ultrapure water. Briefly, 20 µl of CE were mixed with 5 µl of ultrapure water and 150 µl of work solution. The 96 well plate was incubated protected from light for 30 min and the absorbance was monitored at 595 nm with a microplate reader (Bio-Rad iMark). Ascorbic acid was used to perform a standard curve calibration and as reference compound for compare extracts activity. The reducing activity was expressed in Iron (II) sulphate heptahydrate (FeSO$_4$.7H$_2$O) equivalents.

2.3.4. Superoxide anion scavenging activity (SO)

The superoxide anion assay consists in the reduction of phenazine methosulfate (PMS) by nicotinamide adenine dinucleotide (NADH), that consequently react with molecular oxygen leading

to oxygen radical (O_2^-) formation. The oxygen radical generated reduce the nitroblue tetrazolium (NBT) to formazan, which result in dark blue colour.

The assay was performed as follows: a dilution series of CE at different concentrations (0.25 - 3 mg.ml^{-1}) were prepared in phosphate buffer solution (PBS) at pH 7.4. Briefly, 25 µl of CE were mixed with 25 µl of NADH (PanReac AppliChem, Spain) (1.97 mM in PBS at pH 7.4), 75 µl of NBT (Roche, Switzerland) (81.5 µM in PBS at pH7.4) and 25 µl PMS (SIGMA, USA) (16µM in PBS at pH 7.4). The 96 well plate was incubated protected from light at room temperature for 10 min and the absorbance was measured at 570 nm using a microplate reader (Bio-Rad iMark). Ascorbic acid (SIGMA) was used to perform a standard curve calibration and as reference compound for compare extracts activity. The different solutions were prepared daily and kept protected from the light. All measurements were performed in triplicate.

The superoxide scavenging activity was expressed in percentage using the following equation.

$$\% \ Superoxide \ scavenging \ activity$$
$$= \frac{Abscontrol - (Abssample - Absblank)}{Abscontrol} \times 100$$

Abs control- absorbance of NBT, NADH and PMS reaction

Abs sample- absorbance of NBT, NADH and PMS reaction with CE

Abs blank- absorbance of CE without addition of PMS

2.3.5. Nitric Oxide scavenging activity (NO)

Nitric oxide radical can be generated spontaneously *in vitro* from sodium nitroprusside (SNP) in aqueous solution at physiologic pH and interact with oxygen to produce nitrite ions that can be estimated by the Griess reagent.

In order to evaluate the extract NO scavenging capacity, a dilution series of CE at different concentrations (0.5 - 5mg.ml⁻¹) were prepared in PBS. Briefly, 50 µl of CE were mixed with 50 µl of sodium nitroprusside (SNP, SIGMA, Spain) at 10 mM (prepared in PBS) and incubated at room temperature for 2 hours. Then 50 µl of Griess was added to mixture, and after 10 min of incubation, protected from light at room temperature, readings were taken at 595 nm using a microplate reader (Bio-Rad iMark). Griess reagent contains sulfanilamide (Merck, Germany) (1% sulphanilamide in 2% H_3PO_4) and N-(1-Naphthyl) ethylenediamine (NED, SIGMA, USA) (0.1% in in 2% H_3PO_4). Ascorbic acid was used to perform a standard curve calibration and as reference compound for compare extracts activity. The different solutions were prepared daily and kept protected from the light. All measurements were performed in triplicate.

Nitric oxide scavenging activity were expressed in percentage using the following equation:

$$\% \, Nitric \, oxide \, scavenging \, activity$$
$$= \frac{(Abscontrol - Absblankcontrol) - (Abssample - Absblanksample)}{(Abscontrol - Absblankcontrol)}$$

Abs control- absorbance of SNP and Griess reaction

Abs blankcontrol- absorbance of SNP without Griess and CE

Abs sample- absorbance of SNP, Griess and CE

Abs blanksample -absorbance of SNP and CE without Griess

2.4. Antimicrobial assay

The strains selected were *Staphylococcus epidermidis* CECT4183/AtCC35983, *Pseudomonas aureginosa* AtCC10145, *Staphylococcus aureus* AtCC25923, *Escherichia coli* CECT426.

The antimicrobial activity of cladodes extract was evaluated by estimating the minimum inhibitory concentration (MIC) of the extract. MIC is the lowest concentration that inhibits visible growth of the bacteria, after 24h incubation.

The bacteria were inoculated overnight in 10 ml of Lysogeny broth medium. The overnight culture was adjusted at an optical density of to 0.1 at 600 nm. The assay was performed in microplate as follow: 50 µl of a dilution series of CE at different concentrations (0.625 to 20 mg.ml[1]) in LB medium was mixed with 50 µl of LB medium and 50 µl of inoculum (diluted 1:100). To ensure the assay integrity also was performed: a growth control (100 µl of LB medium was mixed with 50 µl of inoculum), a death control (50 µl of chloramphenicol at 120 µg.ml[1] was mixed with 50 µl of LB medium and 50 µl of inoculum) and a DMSO growth control (50 µl of DMSO percentage present in each concentration of cladode extract tested was mixed with 50 µl of LB medium and 50 µl of inoculum).

Further ahead, the microplate was incubated at a temperature of 30 °C and agitation of 200 rpm. For the MIC determination, 50 µl of 2,3,5-Triphenyltetrazolium chloride (TTC) (SIGMA, USA) was added to the microplate, after 1 hour the viable cells metabolized TCC into formazan that had deep red colour which was measured on a spectrophotometer at 570 nm (Bio-Rad iMark). In addition, a measured at 600 nm by the spectrophotometer (SpectraMax Plus 384) without TCC was also performed for the MIC determination.

The MBC (minimum bactericidal concentration) was determinate by removing 50 µl of microplate that was incubated for 24 hours following dilution of 10^{-3} to place 20 µl on a petri dish with LB medium and incubated for 24 hours at 30 °C, the plates that do not show any colonies means bactericidal effect. The method was based on the Clinical and Laboratory Standards Institute (CLSI) protocol (Schwalbe et al., 2007; Balouiri et al., 2016). The MIC was calculated with the following equation:

$$\% \, microbial \, viability \; = \frac{(Abssample - Absblanksample)}{(Abssample(DMSO) - Absblanksample(DMSO))} \times 100$$

Abssample- absorbance of CE and inoculum

Absblank- absorbance of CE and LB medium

Abssample (DMSO)- absorbance of DMSO percentage present in the CE and inoculum

Abs sample (DMSO)-absorbance of DMSO percentage present in the CE and LB medium

2.5. *In vitro* cellular viability assays

2.5.1. MTT assay

The putative cytotoxic effect of cladode extracts was estimated on fibroblasts cell line (NCTC clone 929 [L cell- L929 ATCC-CCL-1] from rats by the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. L929 cells ($1x10^5$ cells.ml^{-1}) were seeded onto 96 well plates, 200 µl of sample at different concentrations were added and cells were incubated. For the assay was prepared an MTT (SIGMA) stock solution of 5 mg.ml^{-1}. The assay was proceeded in the microplate with addition of 200 µl of to each well and of an extract series dilution (1 to 0.05 mg.ml^{-1}) in medium, followed by incubation for 4h and 24h at 37°C. After 100 µl of MTT, 0.5 mg.ml^{-1} were added, and plates were incubated at 37°C for 1h30min. Then, each well was vacuumed and 150 µl of DMSO-methanol (1:1) solution were added to each well, plates were placed into a shaker protected from light for 20 min, formazan absorbance was monitored at 570 nm in a microplate reader (Bio-Rad iMark).

2.6. Cream formulation

The cream formulation preparation was divided into five phases (table 3): firstly, the phases A and B were heated separately until reach 70 °C. At that point, the phase A was added to phase B and emulsified at 500 rpm with a mixture (Silverson L5T), after emulsification, phase D was slowly added. With mixture cooled, phase C was mixed vigorously. Finally, 0.5% of Euxyl® PE 9010 and 1% of essential oil mixture were slowly added. The cream pH was measured, presenting a pH of 5, suitable for skin use. It is important to mention that phase C was prepared: dissolving the cladode extract in zemea, followed by *Aloe vera* and hyaluronic acid addition. A cream using distilled water instead of cladodes extract was made as control.

Table 3. Cream formulation containing Cladodes extract.

Phase	Ingredient	Composition (% w/w)
A	Distilled water	65 %
	Glycerol	2.5 %
	Citric Acid	0.05 %
	Betaine	2 %
B	Vegetable oils	10 %
	Shea butter	1.5%
	Cocoa butter	1.5 %
	Montanov™ 68	3.5 %
	Cetyl alcohol	0.5 %
	Butylated hydroxytoluene (BHT)	0.05 %
C	Hyaluronic acid	0.05 %
	Zemea	1.5 %
	Cladodes extract	0.15 %
	Distilled Water	12.5 %
	Aloe Vera	0.1 %
D	Dermofeel® PA-12	0.8 %
	Xanthan Gum	1.2 %
E	Euxyl® PE 9010	0.5 %
	Essential oil mixture	1 %

2.6.1. Cream stability test

In order to evaluate the stability of creams, 14 g of cream were centrifuged during 30 min at 5000 rpm, twice. Samples were heated at 40 °C during 30 min and centrifuged for one more time. Rheology and morphology of the cream was checked visually before and after treatments.

2.6.2. Microbiological control

In order to certify that cream is not contaminated with bacteria, 1 g of each cream was homogenized with 10 mL of LB (Luria Bertani) and 0.1 ml was inoculated in a petri dish and incubated at 37 °C for 48h. The procedure was repeated 3 times.

2.7. *In vivo* cream evaluation

The *in vivo* evaluation of cladode cream and base cream on human skin was performed in 4 healthy volunteers (3 females and 1 male) with ages between 25 to 36 years. The evaluation of skin volunteers was done in the right inner forearm and upper arm where was delineate three areas for testing the skin with the base cream, cladode cream and untreated (without any cream application) (figure 2). The skin evaluation was performed with three non-invasive probes: Corneometer®, Tewameter® and the Mexameter® from Courage-Khazaka electronic GmbH, Köln, Germany apparatus.
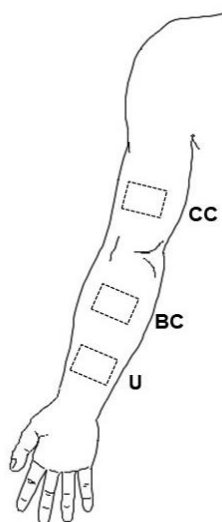
Figure 2. Illustration of the three delineate areas: untreated (U), base cream (BC) and cladode cream (CC).

The Corneometer ® measure the hydration at skin surface (stratum corneum layer) in arbitrary units between 0 and 130, the units bellow 30 means very dry skin, between 30 to 40 means dry and more than 40 means sufficiently moisturized.

The Tewameter® measure the transepidermal water-loss (twel) through the density gradient of the water evaporation from the skin indirectly by the two pairs of sensors (temperature and relative humidity) inside of hollow cylinder. The twel values are expressed in $g/h/m^2$, the values between 0 to 10 means very health condition, 10 to 15 means healthy condition, 15 to 25 means normal condition and 25 to 30 strained condition and above of 30 critical.

The Mexameter® measure the reflectance based on absorption/ reflection from the skin, at wavelengths green and red measure the haemoglobin (erythema) and at wavelengths red and near-infrared measure the melanin. In order to evaluate the possible skin allergy of the cream, only the erythema values are going to be analysed. The erythema state values are represented in table 4.

Table 4. Erythema states and the respective values from the Mexameter® probe.

Erythema state	Values (u.a)
No erythema	0-170
Minimal erythema	170-330
Diffuse redness	330-450
High erythema	450-570
Extreme erythema	Over 570

The measures were taken during the Autumn in order to avoid the excessively sweating (Rim et al., 2005) and UV exposure. The temperature and relative humidity should be constant, the optimal room conditions are 20 °C and 40-60 % relative humidity. Before the measures, volunteers had to rest for at least 10-20 for acclimation with their arms uncovered.

In order to evaluate the hydration with Corneometer ® probe and the erythema with Mexameter® probe a minimum of 5 measures were taken. For Tewameter ® were taken 10

measures after the probe stabilize (30 to 45 seconds or when the standard deviation of 0.2 is reached (stable measurement)). The measures were proceeded as follow:

1- At 0 hours the measures were taken without the influence of any topical product added.

2- At 3 hours with previous cream application.

3- At 6 hours with previous cream reapplication.

The method, efficacy and possible side-effects of this study were explaining to the volunteers and we obtained their consent to proceed.

2.8. Statistical analysis

All values were obtained by triplicate and expressed as mean ± standard deviation (SD). EC_{50} values were obtained using the model log(agonist) vs normalized response—variable slope and differences among means were determined using an unpaired two-tailed Student´s test with high level of significance of $p < 0.05$. Data were analysed performing a one-way and two-way analysis of variance (ANOVA). The statistical analysis was performed in GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California USA.

3. Results and Discussion

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3.1. Phytochemcial analysis

The identification of the phenolic profile of cladodes extract (CE) was based on the interpretation of UV-Vis spectra and retention time of the sample compounds compared with the retention time and UV-Vis spectra of standard compounds and bibliographic research. A typical cladode HPLC chromatogram is presented in figure 3. We were able to identify eight (8) compounds (table 5). The standard compounds are reported in table 6. The majors compounds identified were quercetin derivates, especially peak 5 identified as quercetin-3-glucoside which matched the peak absorbance and the retention time of standard compound (Band II 254 nm and Band I 355 nm; retention time of 31.48). In addition, peaks 6, 7 and 8 could be quercetin-3-rutinoside or quercetin-3—O-glucosyl-rhamnosyl-(xyloside or arabinoside), compounds that were identified by other researchers (Guevara-Figueroa et al., 2010; Ginestra et al., 2009; Astello-García et al., 2015).
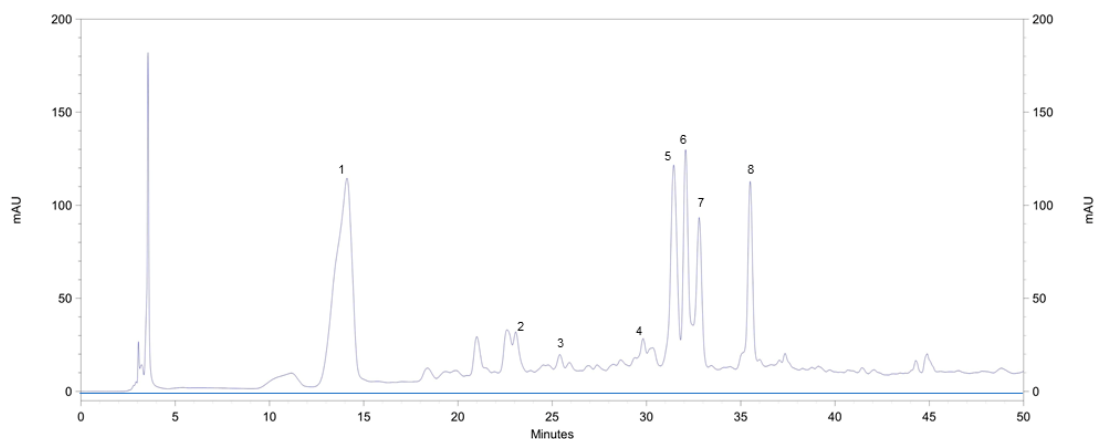
Figure 3. HPLC-DAD chromatogram (280 nm) of cladodes extract. Peak identification, Table 5.

Table 5. Phenolic compounds identified peaks in cladodes extract by HPLC-DAD analysis.

Peak	Retention time (min)	Absorbance (nm)		Compound
		Band I	**Band II**	
1	14.13	-	274	Catechin derivate
2	23.04	323	-	Chlorogenic acid derivate
3	25.40	323	-	Chlorogenic acid derivate
4	29.82	332	-	Flavonoid?
5	31.49	353	253	Quercetin-3-glucoside
6	32.07	353	253	Quercetin derivate
7	32.80	353	253	Quercetin derivate
8	35.54	353	253	Quercetin derivate

Table 6. Standard compounds by HPLC-DAD analysis.

Compound	Retention time (min)	Absorbance (nm)	
		Band I	**Band II**
Gallic acid	6.87	-	270
Chlorogenic acid	20.35	323	-
Caffeic acid	21.36	321	.
Epicatechin	22.70	-	278
Chicoric acid	25.08	327	-
Naringenin	30.31	-	285
Hesperidin	30.81	-	283
Luteolin-7-glucoside	30.81	347	-
Quercetin-3-glucoside	31.48	355	254
Ellagic acid	33.01	366	253
Quercetin	36.54	379	253
Apigenin	38.92	337	-

Lanuzza et al. (2017) reported similar compounds for CE, were catechin appear at first followed by chlorogenic acid. In addition, chlorogenic acid derivates were identified by liquid

chromatography mass spectrometry (LC-MS) analysis by Astello-García (2015). The catechin derivate (peak 1) that had a maximum peak absorbance of 274 nm could be one of following compounds epicatechin gallate (275.7nm) or epigallocatechin gallate (274.5 nm) (Sharma et al., 2011).

The UV-Vis spectra of compound peak 4 resembles a flavonoid but does not match none of the standards injected and was not found support bibliographic for its identification.

From the HPLC-DAD quantification, the cladode extracts accounted for 3.75 mg QE equivalent /g dw biomass of phenolic content. Our phenolic content it is similar with Guevara-Figueroa and collaborators (2010) who quantified 3.1 mg QE/g dw biomass of flavonoid content for several *O. ficus-indica* varieties (*Amarillo, Blanco and Cristalino*). Both method for quantification are different. The method from previous researchers it is directed for flavonoid quantification by measuring the extract absorbance at 365.7 nm. On the other hand, our method by HPLC-DAD showed that our phenolic content it is majority compost by flavonoids which could justify the similarities. The extraction method (solvent polarity, pH and extraction time), plant variety, maturity stage and collecting season are factors that influence the cladodes phenolic content.

3.2. Antioxidants assays

The reactive species of oxygen (ROS) at low or moderate levels are necessary for cellular response and immune system (Pham-Huy et al., 2008). The neutrophils, monocytes, macrophages and eosinophils release ROS as mechanism the defence against harmful agents (Halliwell, 1996). However, an uncontrolled overproduction of ROS leads to oxidative stress that consequently damaged the cells. The antioxidants play an important role at the neutralization of the free radicals excess for the protection of the cells (Pham-Huy et al., 2008).

The ABTS and DPPH assays are widely used for determining the antioxidant activity of phenolic compounds (Nenadis et al., 2004). These assays can measure the capacity of an antioxidant

donates hydrogen to scavenge the radical by forming a stable compound and/or transfer one electron to reduce the radical (Nenadis et al., 2004; Karadag et al., 2009).

The cladode extract exhibited an EC_{50} value of 2.45 ± 0.72 mg.ml^{-1} dwr for DPPH assay, Trolox was also used as a reference compound, which showed EC_{50} of 0.014 ± 0.0007 mg.ml^{-1} (figure 4). This value is higher when compared with other researchers. For example, Lee (2002) performed an extraction 1:10 cladode-ethanol and obtained an EC_{50} of 0.0093 mg.ml^{-1} dwr. Avila-Nava (2014) performed three serial extraction with methanol-water (50/50, v/v), acetone-water (70/30, v/v) and water, followed by an acid hydrolyzation, obtaining an EC_{50} of 0.867 mg.ml^{-1} dwr. Which is in accordance with other *Opuntia* species like *O. monocantha* that showed an EC_{50} of 0.833 mg.ml^{-1} dwr (Valente et al., 2010) and *O. dillenii* showed an EC_{50} of 0.048 mg.ml^{-1} dwr (Qui et al., 2002). The DPPH protocol performed by the other researchers differs from ours. The concentration of DPPH, the volume proportion and the performance in cuvette are factors that influenced the differences between the results previous described in relation to ours. For example, the researchers used DPPH concentrations of 100 µM or 200 µM, which is lower than ours (400 µM). The concentration of 400 µM used lead to higher DPPH radical formation, which required a high CE concentration to scavenge. The extraction method could be another possible reason that explain the differences between the results. This means that factors like temperature, pH, solvent polarity, extraction time and other could yield higher or lower phenolic compounds content modifying the antioxidant power.

In contrast, CE presented an EC_{50} value of 0.23 ± 0.016 mg.ml^{-1} dwr and reference compound Trolox showed an EC_{50} of 0.0044 ± 0.00054 mg.ml^{-1} (figure 5) for ABTS assay. Our ABTS activity seems to present higher activity compared with Petruk and collaborators (2017) which reported an EC_{50} of 0.52 mg.ml^{-1} for cladode extract that was obtained by simple mechanical press. On the other hand, Figueroa-Pérez and collaborators (2018) reported higher activity than ours with

an EC_{50} of 0.0254 mg.ml^{-1}. The differences that could be attributed to cladode extraction process, maturity stage, local growing conditions and/or variety.

The antioxidant power of CE and Trolox on ABTS assay was higher compared with the values of DPPH assay. As was mentioned above, HPLC analysis showed that cladode extract presented higher amounts of quercetin glycosylated, glycosylation increases polarity turning the molecule hydrophilic (Wang and Bi., 2018). DPPH radical is spontaneously formed in organic solutions, hindering hydrophilic molecules from donate a hydrogen or an electron to reduce the DPPH molecule (Arnao, 2000) thus reducing the antioxidant potential. By the other hand, ABTS is performed in aqueous environment, this promotes hydrogen or electron donation of molecules like quercetin glycosylated increasing the antioxidant potential (Arnao, 2000).

Figure 4. EC_{50} values of cladodes extract (CE) and Trolox (T) for DPPH assay. Each bar represent mean ± SD. The values are significantly different (P≤0.0001), statistically analyses by t-test. The results are obtained with a minimum of three independent assays.
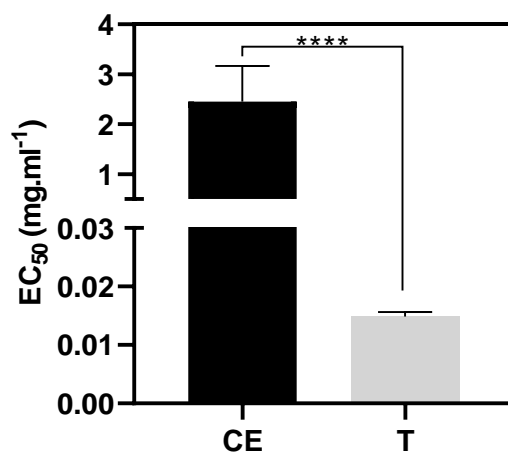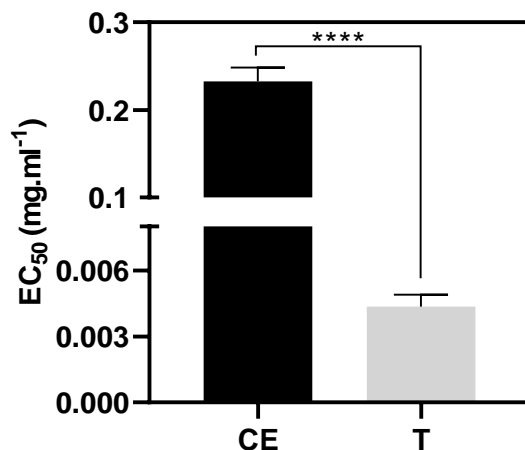
Figure 5. EC_{50} values of cladodes extract (CE) and Trolox (T) for ABTS assay. Each bar represent mean ± SD. The values are significantly different (P≤0.0001), statistically analyses by t-test. The results are obtained with a minimum of three independent assays.

Iron is present in the structure of many enzymes and proteins. The combination of iron availability with the increase of reactive oxygen species such as H_2O_2 and $O_2^-\cdot$ promote the oxidative cellular damage (Meneghini, 1997). For example the H_2O_2 reaction with metals such Fe^{2+} leads to the formation of the most dangerously radical the HO· responsible for DNA damage like the production of 8-oxo-2′-deoxyguanosine (Matés et al., 1999). The CE for the iron-chelating assay exhibited an EC_{50} of 1 ± 0.29 mg.ml^{-1} dwr, EDTA also was used as a reference chelator which showed an EC_{50} of 0.0058 ± 0.0011 mg.ml^{-1} (figure 6). From HPLC analysis, we could identify catechin and quercetin derivates in high amounts (figure 3). Flavonoid compounds with a catechol ring B, the 3-hydroxyl group, the 4-keto group and the 2,3-double bond conformation provide high iron chelation power (figure 7) (Mladěnka et al., 2011; Khokhar and Apente, 2003). The catechin compound had some of the characteristics previous describe (3-hydroxyl group and catechol ring B) but some of the derivates of catechin like epigallocatechin or epicatechin gallate are related with the low iron binding efficiency (Khokhar and Apente, 2003). Quercetin had all the characteristics (figure 8) being more effective than catechin but also the quercetin derivates that are glycosylated showed lower chelation power because of the 3-OH group alteration (figure 9) (Mladěnka et al., 2011; Khokhar and Apente, 2003).
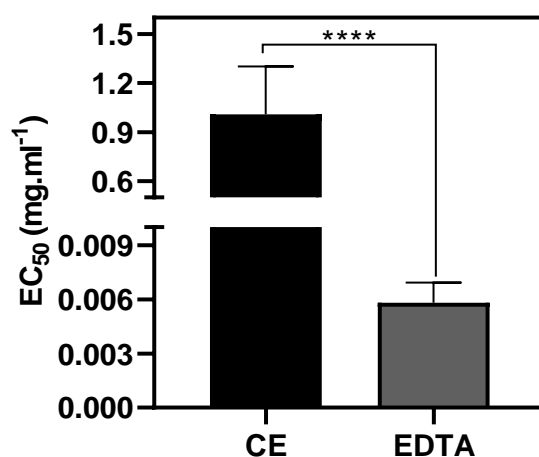
Figure 6. EC_{50} values of cladodes extract (CE) and EDTA-Na_2 (EDTA) for ICA assay. Each bar represent mean ± SD. The values are significantly different (P≤0.0001), statistically analyses by t-test. The results are obtained with a minimum of three independent assays.
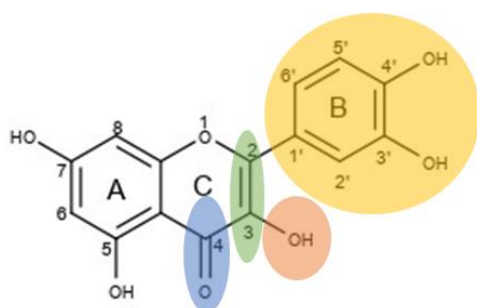
**Figure 7.** Characteristics of flavonoids that confer higher chelation power: the catechol ring B (yellow shaded), the 2,3-double bond (green shaded), the 3-hydroxyl group (red shaded) and the 4-keto group (blue shaded).
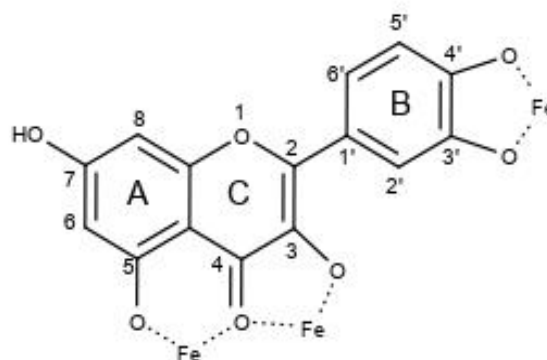
Figure 8. Possible sites for iron binding in the quercetin molecule. Adapted from Procházková and collaborators (2011).

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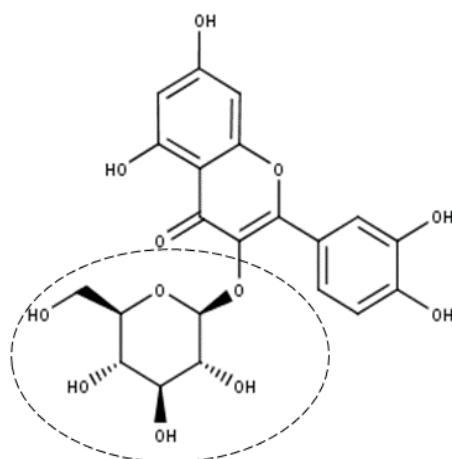
Figure 9. Quercetin-3-glucoside. Delineated substitution in 3-OH group by a glycosyl group.

FRAP assay is based on electron transfer of a substrate to reduce Fe^{3+} into Fe^{2+} (Huang et al., 2005). This method only evaluates the substrate reductant ability, which may reflect their capacity in reducing reactive species (Karadag et al., 2009). The CE showed ferric reducing activity of 71.22 ± 3.15 μM Fe (II)/mg dwr which is lower compared with the reference compound ascorbic acid (figure 10). According to Firuzi and collaborators (2005) compounds like quercetin, fisetin and myricetin showed high ferric reducing power, flavonoids that possess the structures mentioned above proving once more time these structures role in the antioxidant power. As previously referred, our CE had higher amounts of quercetin glycosylated that might also compromise the ferric reducing power.
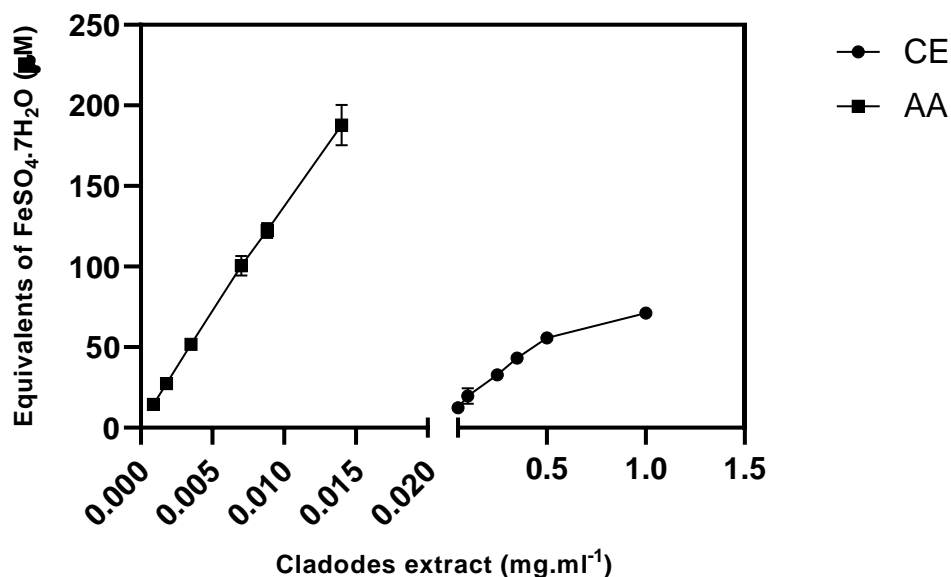
Figure 10. FRAP values of cladodes extract (CE) and Ascorbic acid (AA) express in equivalents of $FeSO_4.H_2O$. Each graph point represent mean ± SD. The results are obtained with a minimum of three independent assays.

Despite the superoxide anion is a weak oxidant, it can generate dangerous hydroxyl radicals and singlet oxygen (Alam et al., 2013), causing peroxidation in the polyunsaturated fatty acids leading to the rupture of cell membranes (Huguet et al., 1990). For the superoxide assay, the CE exhibited an EC_{50} of 2.30 ± 0.22 mg.ml^{-1} dwr, Ascorbic acid also was used as a reference compound, which showed an EC_{50} of 0.63 ± 0.13 mg.ml^{-1} (figure 11). Avila-Nava and collaborators (2014) showed an EC_{50} of 1.874 mg.ml^{-1} dwr which it is not too far from ours.
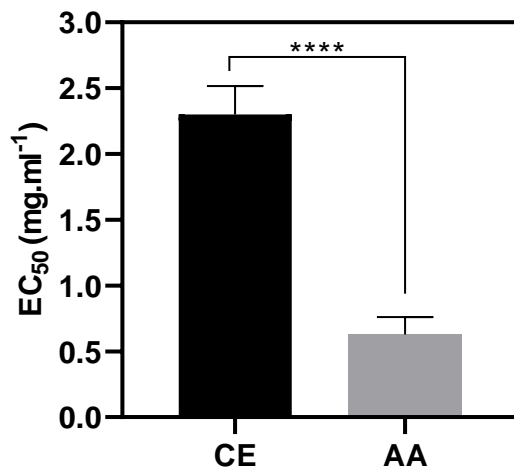
Figure 11. EC_{50} values of cladodes extract (CE) and ascorbic acid (AA) for superoxide assay. Each bar represent mean ± SD. The values are significantly different (P≤0.0001), statistically analyses by t-test. The results are obtained with a minimum of three independent assays.

The $NO^{.}$ radical has an important role in many physiological processes mediating vasodilation, neurotransmission and neutrophil killing of pathogens. On the other hand, the overproducing of $NO^{.}$ induce tissue damage (Halliwell, 1996). In addition, the $NO^{.}$ reaction with $O_2^{.-}$ lead to peroxynitrite formation a cytotoxic species that also damaged the tissues (Halliwell, 1996). In the oxide nitric (NO) assay, the CE exhibited an EC_{50} of 4 ± 0.43 mg.ml^{-1} dwr, Ascorbic acid also was used as reference compound, which showed an EC_{50} of 0.25 ± 0.035 mg.ml^{-1} (figure 12). The flavonoids can scavenge NO directedly but also are capable to inhibit several pro-inflammatory mediators that are involve on NO production like interleukin (IL)-1b, tumour necrosis factor (TNF)-a, and lipopolysaccharide (LPS) (Procházková et al., 2011; Matsuda et al., 2003). Lee and collaborators (2006) showed through cell line that cladode extracts had anti-inflammatory activity by inhibiting NO production, reducing the inducible nitric oxide synthase (iNOS) expression and suppressing the degradation of of I-κB-α protein. Despite the CE showed lower oxide nitric scavenging, it may act on the pro-inflammatory mediators as previous mentioned.
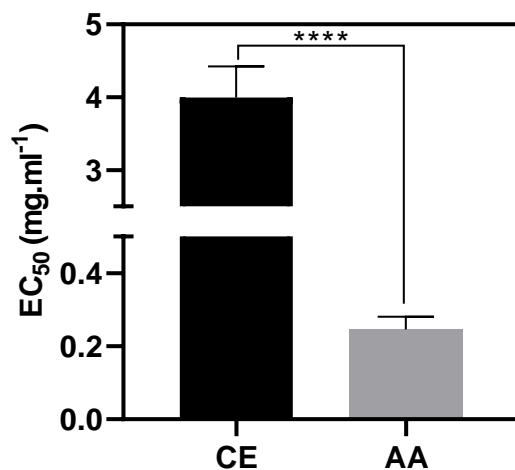
Figure 12. EC_{50} values of cladodes extract (CE) and ascorbic acid (AA) for nitric oxide assay. Each bar represent mean $\pm$ SD. The values are significantly different (P$\leq$0.0001), statistically analyses by t-test. The results are obtained with a minimum of three independent assays.

The lower antioxidant power of CE in the previous assays might be due to the quercetin glycosylation. The substitution of 3-hydroxil group in the ring C by a glycoside lead to a reduction of antioxidant power (Procházková et a., 2011; Heim et al.,2002). The 3-hydroxil conferee planarity to the flavonols (catechin) and the flavanols (quercetin) inducing conjugation, electron dislocation which increase in flavonoid phenoxyl radical stability (figure 13) (Heim et al., 2002). Furthermore, in the realization of antioxidants assays, the type of solvent and pH of the assay solution influence significantly the electrons exchange (Abramovič et al., 2018).
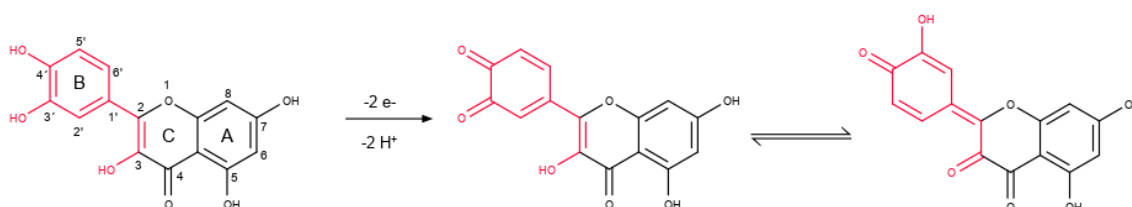
Figure 13. Quercetin stabilization into two highly stable molecules as a result of bioelectronic oxidation. The presence of a free 3-OH group its essential to donate a proton for the conjugation of the aromatic rings. Adapted from Firuzi and collaborators (2005).

The term antioxidant is defined as "any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that

substrate" (Halliwell and Gutteridge, 1995). Despite the low antioxidant power in the majority of antioxidants assays CE demonstrated a low EC_{50} of 0.23 mg.ml^{-1} for the ABTS assay, as was explained before is due to the hydrophilic antioxidants. These antioxidant molecules with hydrophilic characteristics in the aqueous domain can also prevent the lipid oxidation caused by initiators that also cause oxidative damage in hydrophobic region of the lipid bilayer (Verstraeten et al., 2003). Considering what was stated early, CE might confer some protection to the skin against ROS which still validates their possible incorporation in a cosmeceutical.

3.3. Antimicrobial assay

During the antimicrobial assay, one of the biggest problems was the solvent (DMSO) used to resuspended CE since is toxic for bacteria at a certain percentage. As a result of DMSO toxicity and the high concentrations required to identify a MIC value for the strain *E. coli*, 1 g of cladode extract was resuspended at the lowest quantity of DMSO possible of 3 ml. Therefore CE at 20 mg.ml^{-1} during 24h of incubation with *E. coli* showed 4.0×10^5 CFU.ml^{-1} and DMSO at 5 % (percentage of DMSO present in the extract at 20 mg.ml^{-1}) showed 3.0×10^9 CFU.ml^{-1}, ensuring the low significant interference of DMSO on MIC determination at this percentage.

For MIC determination with a colorimetric method, *E. coli was* incubated for 24 hours with several CE concentrations, and then tetrazolium red (TCC) was added to the microplate and incubated for 1h. Tough this method showed a problem, at a CE concentration of 8 mg.ml^{-1} the medium did not show any red colour (no bacteria metabolic activity), which indicates our MIC. However, after 24h the extract also showed visible turbidity, suggesting that some compound precipitation could occur. The initial inoculum added to the microplate had 3.23×10^6 CFU.ml^{-1}, and the inoculum with CE concentration of 8 mg.ml^{-1} after 24h of incubation was 4.28×10^8 CFU.ml^{-1}, which showed that CE at 8 mg.ml^{-1} did not have an inhibitory effect.

Moreover, MIC determination by measuring the OD at 600 nm without tetrazolium red was adopted, which was 25 mg.ml^{-1} for the strain *E. coli*. By the other hand, the strain *S. epidermidis* produce biofilm during its growth, this characteristic hindered the MIC determination. In the growth control the bacteria were aggregated being not possible to measure the OD correctly. Furthermore, due to the high concentrations also required to determinate the MIC of other strains (*P. aeruginosa* and *S. aureus*) we acknowledge that cladodes extract not possess significant antimicrobial effects for the concentrations tested.

According to Ginestra and collaborators (2009), the cladode ethanolic extracts fractions did not show inhibitory activity against *E. coli* and *S. aureus*. Wu and collaborators (2013) demonstrated that more hydrophobic flavonoids showed better antibacterial activity being able to permeate the membrane turning the membrane rigidity. For example, the addition of galloyl moiety in the epicatechin derivates such as epicatechin gallate and epigallocatechin enhance their antibacterial activity being more hydrophobic. Despite the fact, that a catechin derivate that could be epicatechin gallate or epigallocatechin was identified, the cladode extract did not show antibacterial activity. On another hand, the cladode extract showed high amounts of quercetin glycosylated specially quercetin-3-glucoside that is a more hydrophilic molecule, and so with ineffective antibacterial activity.

3.4. Cytotoxic effect

Fibroblasts are an area of skin connective-tissue that synthesises collagen, essential for the architectural framework of the body (Alberts et al., 2002), having major role in the healing of damaged tissue producing higher amounts of collagenous matrix (Alberts et al., 2002). The cladodes extract did not show cytotoxic effect on fibroblast l929 cellular line at any tested concentration used (50-1000 μg.ml^{-1}), either at 4 or 24 h of incubation (figure 14 and 15). Instead, it seems that at concentrations of 500 and 250 μg.ml^{-1} exhibit a slightly proliferative effect after 24h (figure 15). The

proliferative effect may be explained by the interaction of membranes with flavonoids compounds. Tsuciya (2010) showed that quercetin increases membrane toughness causing antiproliferative effect on cells, by the other hand, glycosylation of quercetin, especially quercetin3-glucoside did not modify the membrane fluidity. Furthermore, the hydrophilic flavonoids interact at water-lipid interface of membranes establishing hydrogen bonding with the polar head groups of phospholipids protecting the bilayer from oxidative damage and preserving membrane integrity (Erlejman et al., 2004).
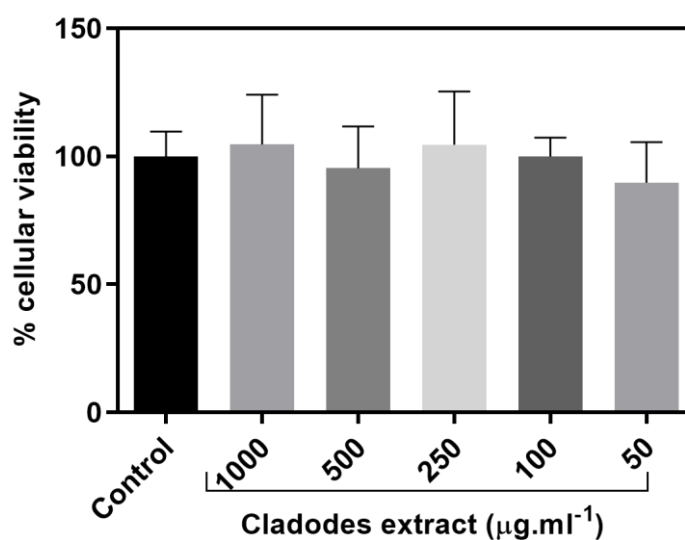
Figure 14. Cellular viability of fibroblast cells line with 4 hours incubation of cladodes extract at several concentrations. Each bar represent mean ± SD. The results are obtained with a minimum of three independent assays. No significant differences were observed among columns. Statistic was analysed by one-way ANOVA.
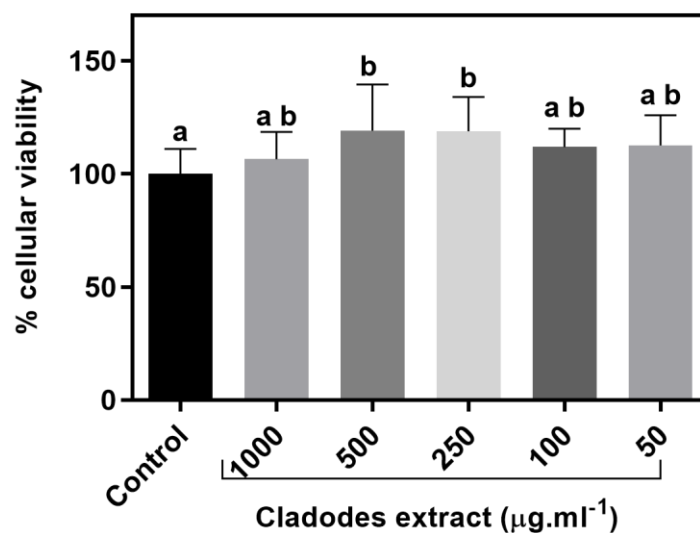
Figure 15. Cellular viability of fibroblast cells line with 24 hours incubation of cladodes extract at several concentrations. Each bar represent mean ± SD. The results are obtained with a minimum of three independent assays. There are significant differences of P≤ 0.01. Statically analyse by one-way ANOVA.

3.5. Cream formulation and in vivo cream evaluation

Based on the results of the antioxidants assays and their lack of significant cytotoxic effect, 1.5 mg/g of cladodes extracts was used for the cream formulation to reach a maximum concentration of 0.15% of CE in the cream. Usually, in cream formulations, the plant extracts content is in a range from 0.1 % to 5 %(Campanini et al., 2013; Da'Belo et al., 2006; Noriega et al., 2015; Re et al., 2009; Sharif et al., 2014; Waqas et al., 2010) , on the other hand, it is important to aware of choosing a safe concentration because of some compounds present in plants extracts can be allergenic, like eugenol, linalool, d-limonene, coumarin, and citronellol (Unitis, 2020)).

The principle of an emulsion is based on two or more immiscible liquid in which one of the liquids is dispersed in the other as small (0.1–100 μm) spherical droplets (McClements, 2015). During the emulsion stabilization, the large oil droplets are fragmented into to small particles. If is correctly emulsified the small oil particles possess low tendency to increase in size and consequently

to separate (Kowalska et al., 2015). In the stability test through centrifugation, no separation of phases was observed both in the base cream and in the cladode cream.

Cosmetics products can be contaminated during the manufacture process, besides, higher water content makes cosmetics highly susceptible to contamination (Lundov et al., 2009). The Scientific Committee on Consumer Products require in a finished cosmetic product, a total viable count for aerobic mesophilic microorganism not exceeding 1000 CFU.g[1] in 0.1 g of cream, as well as, *P. aeruginosa*, *S. aureus*, and *C. albicans* not be detectable (Lundov et al., 2009). The cladode cream and base cream examined for potential contamination showed no microbial growth. The preservatives used in the formulation proved to be adequate for microbiological control. The microorganism contamination leads to organoleptic alterations such as colour and odour, besides composition changings and possibly health issues to the consumers (Lundov et al., 2009). During the *in vivo* cream evaluation, measures were taken with ambient temperatures between 21-16 °C and relative humidity between 47-63 %. The Corneometer® principle is based on the capacitance method, the probe transmits an electromagnetic field that penetrates the skin surface (stratum corneum layer) and the water present in stratum corneum reflects the electromagnetic field that is detected by the probe (Rosado et al., 2009; Chaves et al., 2014). The results of hydration are represented in figure 16. It is important to point out that the skin of each tested person was analysed separately due to the intrinsic differences of the skin.

Figure 16. Measurement of hydration (u.a) in the 3 different areas (untreated (U), base cream (BC) and cladode cream (CC)) at 0, 3 and 6 hours of each volunteer (Person 1, Person 2, Person 3 and Person 4). Each bar represent mean ± SD. There are significant differences from P≤0.05, statistically analyses by Two-Away Anova.

The hydration levels of the untreated (U) areas did not show significant differences, whit exception of person 4 that demonstrated a hydration increase after 6h. This can be explained by metabolism alterations in response to stress or environmental conditions, such as temperature and physical effort that consequently increased the sweating behaviour. On the other hand, the area treated with base cream (BC) revealed a significant increase since the first application, except person 2 (28 % person 1, 26 % person 3 and 13 % person 4). After the second application, persons 1 and 2 did not show significant difference contrarily persons 3 and 4 that registered an increase of 19 % and 24 % respectively. Areas treated with cladode cream (CC) showed different behaviour between persons. For the person 2, no significant differences were observed during the treatment. Besides, after the first application, person 3 and 4 showed a significant increment of 42 % and 22 %,

respectively, and after re-application person 3 demonstrated an increment of 25 % and person 4 a drop 8 %. Person 1 only showed a significant difference after the second application (13 %). Overall, both creams showed hydration effect on the skin, despite that in some cases the skin conditions still dry/very dry, suggesting that may be necessary a prolongation of the treatment to obtain a skin sufficiently moisturized.

The Tewameter® evaluate the transepidermal water loss (twel) that is the water evaporation through the stratum corneum and indicate is integrity (Rim et al., 2005; Wakeman, 2017). There are external factors that influence the twel, such as airflow and direct light radiation. In addition, the increase in skin temperature leads to twel increment as well as the biorhythm (Rim et al., 2005). The results of the twel are represented in figure 17. All the volunteers presented a very healthy skin condition with values below 10 $g/m^2.h$.

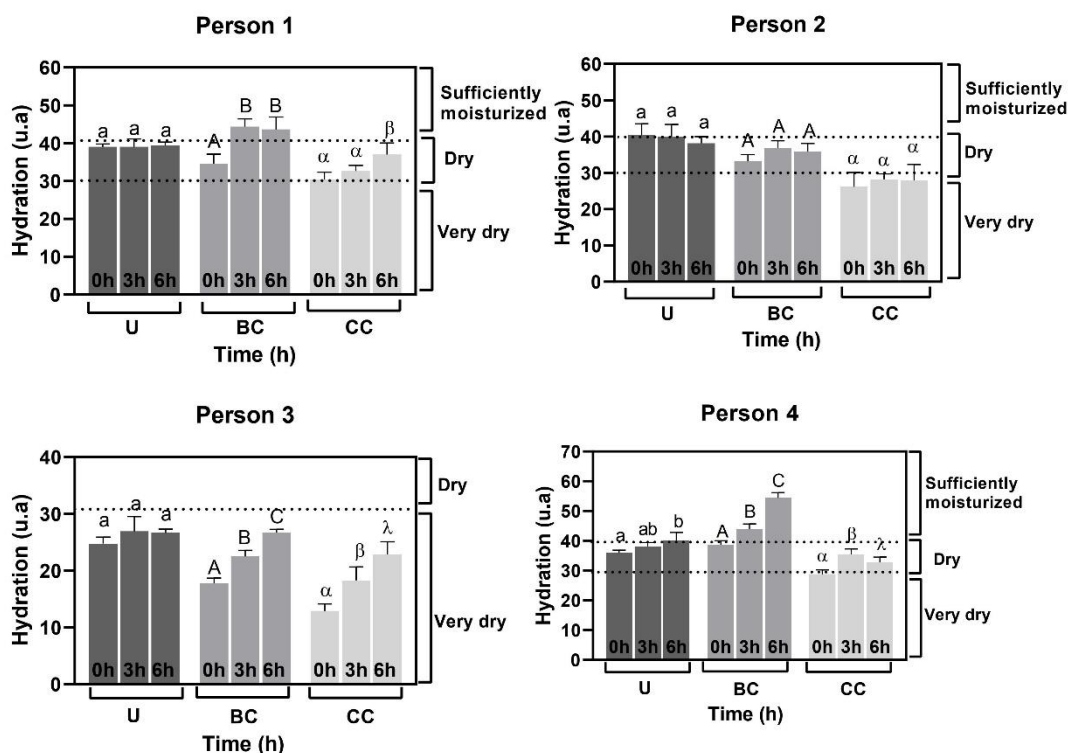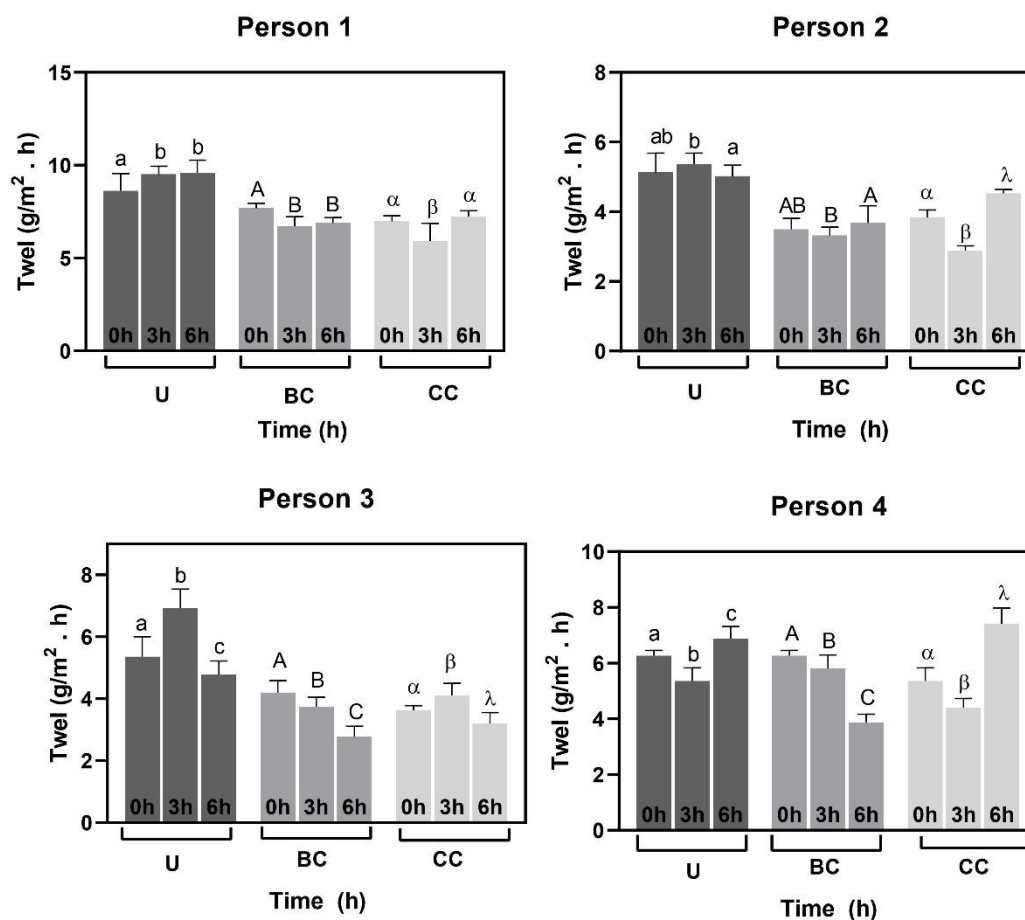**Figure 17.** Measurement of transepidermal water loss (twel) g/m².h in the 3 different areas (untreated (U), base cream (BC) and cladode cream (CC)) at 0, 3 and 6 hours of each volunteer (Person 1, Person 2, Person 3 and Person 4). Each bar represent mean ± SD. There are significant differences from P≤0.05, statistically analyses by Two-Away Anova.

The twel values during the treatment did not show a linear behaviour between the persons and the hours, which difficult the evaluation of the cream effect in transepidermal water loss. The untreated area showed significantly differences to all the persons, which indicates that external factors were a stronger influence in the measurements, as well as, the management with the probe applying pressure, could disturb the blood circulation. On the other hand, the tight contact with skin is needed to prevent water evaporating through gaps between probe and skin (Anthonissen et al., 2013). However, it is possible to observe a potential occlusion effect of both creams. Persons 1, 3

and 4 shows a significantly decrease of twel value after first application. The same was observed for person 1, 2 and 4 with cladode cream treatment.

The Mexameter® measures the content of melanin and haemoglobin (erythema) in the skin. The main objective with this probe was to evaluate if the base cream and cladode cream cause allergy to the skin, for that reason only the erythema values were analysed. The results of erythema are represented in figure 18. The erythema values are individual for each person, according to manual (Courage-Khazaka electronic GmbH, Köln, Germany) the erythema values between 0-170 means no erythema and 170-330 means minimal erythema, in our study, there are healthy skin persons with values above 170 and no visible erythema was observed which could be normal values of their healthy skin.
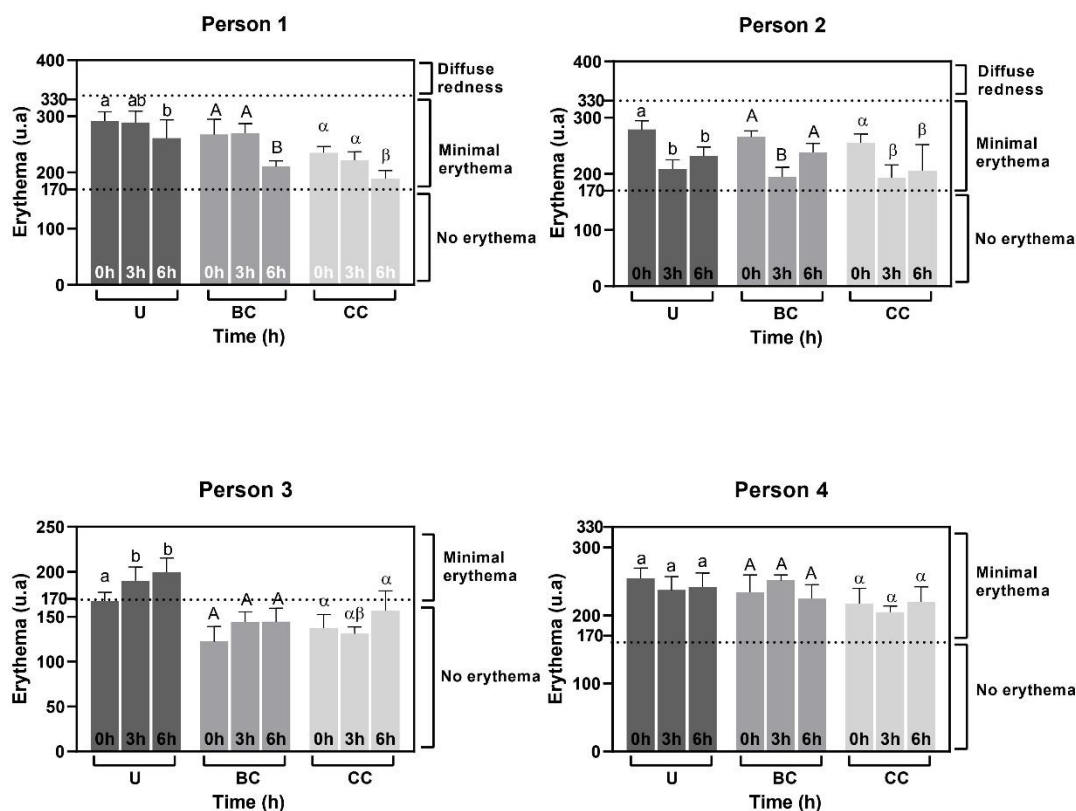
Figure 18. Measurement of erythema level (u.a) in the 3 different areas (untreated (U), base cream (BC) and cladode cream (CC)) at 0, 3 and 6 hours each volunteer (Person 1, Person 2, Person 3 and Person 4). Each bar represent mean ± SD. There are significant differences from P≤0.05, statistically analyses by Two-Away Anova.

The erythema values during the treatment did not show a linear behaviour, the area untreated shows ambiguous variations for all the persons, except for person 4 that demonstrated no significant differences to all areas. Blood flow alterations (vasodilation and vasoconstriction) caused by factors like temperature, physical and mental activity exert the greatest influence on skin colour (Fullerton et al., 1996). These mentioned factors could have influenced the results causing abnormal variations and not be related to the influence of the base cream or cladode cream. Overall, we can observe that base cream and cladode cream did not show an increment of erythema level on skin comparing with initial measures without previous cream application.

Other researchers realized a cream formulation containing *Opuntia ficus-indica* extract and an *in vivo evaluation* of the cream with probes Corneometer® and Tewameter®. Ribeiro and collaborators (2015) performed an hydroglycolic (20:80 of water/propylene glycol) extraction from fresh cladodes with a ratio of 1:3 plant-solvent. The cream formulated with cladode hydroglycolic extract showed an increment of more than 20 % on hydration levels, however, the twel levels increased until 5h. Damasceno and collaborators (2016) performed the same hydroglycolic extraction that previous researchers, the hydration levels of cream with cladode hydroglycolic extract showed to be less effective. The twel values level showed a decrease at 1h and a continuous increase until the 5h. The extracts of previous researchers differ from our hydroalcoholic extract, being rich in mono and polysaccharides which confers humectants properties. The cladode cream formulated by the Ribeiro and collaborators (2018) showed higher capability of hydration compared with our cladode cream, on the other hand, Damasceno and collaborators (2016) showed similarity capability of hydration. Ribeiro and collaborators (2015) acknowledge that their cream only showed humectant properties and no occlusion effect.

The topical application of cosmetics to improve skin condition is considered important and their actual effects must be scientifically investigated and documented (Rim et al., 2005). The stratum corneum layer possesses the capability of retaining water, playing an important role as a barrier for the transepidermal water loss (twel) (Verdier-Sévrain and Bonté, 2007). A healthy skin possesses in the stratum corneum layer a water content higher than 10 % (Berardesca et al., 1997; Verdier-Sévrain and Bonté, 2007). The water content is essential for the hydrolytic enzymatic processes required for normal desquamation, a level below of 10 % leads to adhesion and accumulation of corneocytes on the skin surface, arising a visible appearance of dryness, roughness, scaling, and flaking (Verdier-Sévrain and Bonté, 2007).

4. Conclusion and Futures Perspectives

4.   Conclusion and futures perspectives

The topical application of natural ingredients has been used for millennia in traditional medicine. In the last 20 years, scientific studies reported the benefits of natural ingredients on the skin (Fowler Jr et al., 2010). The plants extracts are a source for the natural obtention of bioactive compounds. Therefore, it is important to be aware of the extraction methods, plant-to-solvent ratios and the content of bioactive compounds (Ribeiro et al., 2015). Also, the *in vitro* studies of antioxidant activity, antimicrobial activity and cytotoxicity activity are important for the potentiality evaluation of a plant extract incorporation in cosmeceutical. For complete the admission of cosmeceutical, the *in vivo* assessment is fundamental for report their actual effect on the skin such as hydration, skin rehabilitation and allergenic effect.

Overall, the cladodes extract showed to be less effective than the standard compounds used for each antioxidant assay. From all antioxidants methodologies tested, ABTS assay showed an EC_{50} value of 0.23 mg.ml^{-1} exhibiting the highest antioxidant power. The HPLC analysis demonstrated that cladodes extract had higher amounts of quercetin glycosylated which are more hydrophilic compounds with lower antioxidant power than purified quercetin. Also, this hydrophilic aspect of quercetin glycosylated could lead to advertising in permeate the cellular membranes reflected in non-antimicrobial effect.

In the cellular viability assay with fibroblasts L929 cell line, the cladodes extract did not show a cytotoxic effect, furthermore, might possess a slightly proliferative effect at concentrations 500 and 250 µg.ml^{-1} after 24h incubation. Although the cladode extracts only had a low EC_{50} in the ABTS assay, therefore it could be interesting to study in the fibroblast cell if the cladode extract possesses protective effect, as previous explained the hydrophilic compounds could establish a connection with polar heads of phospholipid bilayer forming protective barrier against ROS.

Trough the *in vivo* cream evaluation, it is not possible to conclude that cladodes extract incorporation in the cream is more effective than the base cream at hydration and twel levels, furthermore, none of both creams showed to be allergenic. However, for supporting this *in vivo* evaluation it is necessary to collect more volunteers to have more database for diminish the intrinsic skin difference.

Further research could explore cladode extract through *in vitro* studies with keratinocytes and fibroblast cell lines to evaluate the protective effect against oxidative insult with H_2O_2 or *tert*-Butyl hydroperoxide (t-BHP). Furthermore, evaluation of the protective effect against UVA-radiation on keratinocytes cells and the wound healing through the measure of the migration distance of fibroblast cells scratched, a method described by Pitz and collaborators (2016). Besides, could be interesting to study if cladodes stimulate collagen production that is also involved in wound healing. It is evident that UV radiation is the major cause of skin ageing trough ROS production having a damaging impact in the connective-tissue, expressly, a decreased of fibroblasts number, collagen and elastin that consequently cause skin ageing (Rittié and Fisher, 2002). Regarding what was mentioned new *in vivo* evaluation could be done this time with more volunteers with a wide range of ages focused on wound healing treatments.

Moreover, there are skin diseases where ROS production is involved, namely atopic dermatitis, psoriasis, irritant contact dermatitis and acne. The ROS generation is a pro-oxidant stimulate of the disease physiologic and pathologic mechanism (Briganti and Picardo, 2003). This issue is also important to explore the development of cosmeceuticals that attenuates the constraints of the diseases, which seriously affect the people quality of life. Cladode extract showed to had antioxidant activity and their incorporation in a cosmeceutical for the treatment of skin diseases could be promising for future research.

5. References

5. References

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