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PATRÍCIA MARQUES DE FARIAS

NOPAL CLADODES FLOUR (*OPUNTIA FICUS-INDICA*): CHARACTERIZATION AND APPLICATION AS A NEW REINFORCEMENT AND ANTIOXIDANT BIOPOLYMER ON STARCH-BASED FILMS.

FORTALEZA
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Advisor: Prof. Dr. Lucicléia Barros de Vasconcelos (UFC).

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To God Almighty.

To the memory of my grandmother, Maria Marques Nonato.

To my sisters, Brenda e Gleiciane.

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ABSTRACT

This thesis studied important issues related to the incorporation of the nopal (*Opuntia ficus-indica*) cladodes flour (NC) in a cassava starch matrix for the development of a sustainable plastic film. Thus, this study aimed to evaluate the influence of the alkaline treatment applied to NC on the film properties as well as to compare the functional efficacy of NC to propolis extract and lignin addition. NC was obtained by grinding and sieving process. The resulting fractions (NC-45, NC-80 and NC-120 mesh sieve) were characterized in terms of chemical composition (XRD and FT-IR), morphology (SEM), content of total phenolics and antioxidant activity (DPPH and ABTS). The FTIR spectra of the NCs showed bands related to the presence of proteins, fibers, lipids, sugars and pectin. The presence of crystallites related to calcium salts, cellulose and hemicellulose peaks were evidenced by XRD. Due to the morphological and chemical characteristics, NC-120 was the chosen fraction. The NC-120 was subjected to an alkaline treatment at different pHs (7, 8, 9, 10, 11 and 12) and subsequently added to the polymeric matrix of cassava starch; glycerol added as a plasticizer. The presence of NC-120 affected starch-starch interactions (SEM) and the thermal stability (DSC). NC-120 incorporation was compared to propolis extract and lignin performance. According to multivariate statistics, NC-120 affected the mechanical properties in a similar way to the addition of lignin and propolis extract, however NC-120 was better incorporated into the cassava starch matrix, providing films with more hydrophobic surface and greater antioxidant activity, attesting its potential as a reinforcing and antioxidant biopolymer (88.43% radical inhibition by the DPPH method). The films obtained did not present antimicrobial activity, nevertheless due to the high antioxidant activity and hydrophibicity modification capacity, NC-120 has a high potential for incorporation in active packaging systems.

Keywords: Nopal (*Opuntia ficus-indica*). Propolis. Lignin. Cassava starch film. Antioxidant activity.

RESUMO

Esta tese estudou questões importantes relacionadas à incorporação da farinha de cladódios (NC) de nopal (*Opuntia ficus-indica*) em uma matriz de amido de mandioca para o desenvolvimento de um filme plástico sustentável. Assim, este estudo teve como objetivo avaliar a influência do tratamento alcalino aplicado a NC nas propriedades do filme, bem como comparar à eficácia funcional da adição da NC a adição do extrato de própolis e lignina. A NC foi obtida pelo processo de moagem e peneiramento. As frações resultantes (NC-45, NC-80 e NC-120 mesh) foram caracterizadas quanto à composição química (DRX e FT-IR), morfologia (MEV), teor de fenólicos totais e atividade antioxidante (DPPH e ABTS). Os espectros de FT-IR das NCs mostraram bandas relacionadas à presença de proteínas, fibras, lipídios, açúcares e pectina. A presença de cristalitos relacionados a sais de cálcio, picos de celulose e hemicelulose foram evidenciados por DRX. Pelas características morfológicas e químicas, NC-120 foi a fração escolhida. A NC-120 foi submetida a um tratamento alcalino em diferentes pHs (7, 8, 9, 10, 11 e 12) e posteriormente adicionado à matriz polimérica de amido de mandioca; o glicerol adicionado como plastificante. A presença da NC-120 afetou as interações amido-amido (SEM) e a estabilidade térmica (DSC). A incorporação da NC-120 foi comparada ao desempenho do extrato de própolis e lignina. De acordo com a estatística multivariada, a NC-120 afetou as propriedades mecânicas de forma semelhante à adição de lignina e extrato de própolis, porém o NC-120 teve a melhor incorporação à matriz do amido de mandioca, proporcionando filmes com superfície mais hidrofóbica e maior atividade antioxidante, atestando seu potencial como biopolímero antioxidante (88,43% de inibição do radical pelo método DPPH) e de reforço. Os filmes obtidos não apresentaram atividade antimicrobiana, porém devido à alta atividade antioxidante e da característica hidrofóbica, a NC-120 possui alto potencial para incorporação em sistemas de embalagem ativa.

Palavras-chave: Nopal (*Opuntia ficus-indica*). Própolis. Lignina. Filmes de amido de mandioca. Atividade antioxidante.

ABBREVIATION LIST

ABTS	2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
ATP	Adenosine triphosphate
DPPH	2,2-diphenyl-1-picryl-hydrazyl-hydrate
E	Elongation
DSC	Differential scanning calorimetry
FAO	Food and Agriculture Organization of the United Nations
FTIR	Fourier-transform infrared spectroscopy
GAE	Gallic acid
GRAS	Generally recognized as a safe substance
HPMC	Hydroxypropyl methylcellulose
HSQC	Heteronuclear single quantum coherence, 2D-NMR
L	Lignin
LNP	Lignin nanoparticles
MY	Young's modulus
N, NC	Nopal cladode flour (*Opuntia ficus-indica*)
N12	Nopal cladode flour treated with pH 12
NMR	Nuclear magnetic resonance
P1	Propolis extract addition concentration 1
P2	Propolis extract addition concentration 2
PLA	Polylactic acid
ROS	Reactive oxygen species
S	Starch

SEM	Scanning Electron Microscope
TPS	Thermoplastic starch
TS	Tensile strenght
TSA	Tryptone soy agar
WCA	Water contact angle degree
WVP	Water vapor permeability
ZnO	Zinc oxide

CONTENT

1 INTRODUCTION

Since 1950, when it was produced on a large scale, and mainly after the Second World War, the manufacture of plastic has grown extraordinarily and has significantly outperformed other materials, such as glass and metal (Geyer, Jambeck & Law, 2017). Most of the materials used in the manufacture of food packaging come from synthetic polymers of petrochemical origin, a resource considered non-renewable and non-biodegradable, which persist for many years after disposal (Cerqueira et al., 2016; Podshivalov et al., 2017). Synthetic polymer waste contributes to serious environmental pollution problems and, although recycling is one of the most popular alternatives in the world, the process involves high energy consumption and it is not possible to recover 100% of the waste produced, which ends up being burned or landfilled (Parra et al., 2004).

In an attempt to reduce the environmental impact caused by the plastic industry, natural polymers, or biopolymers, from renewable resources have been extensively investigated in recent years, with the aim of developing new biodegradable materials from natural resources (Sessini et al., 2016).

Starch, for example, is a renewable biopolymer and a low-cost production material that can be obtained from corn, potatoes, cassava and other crops. Starch is biodegradable, colorless, non-toxic, and edible and has a good gas barrier, making it a good option for use in edible coatings and films (Mali et al. 2004). All starches contain amylose and amylopectin, in proportions that vary according to the source of the starch; and this natural variation provides different properties to the material (Gross & Kalra, 2002). Cassava starch is recognized as a safe substance (GRAS), abundant in Brazil and occupies a prominent position in the Brazilian economy, as the activities related to the cultivation of this plant and its derivatives generate almost one million direct jobs in Brazil (Alves, Junior & De Campos, 2010).

Despite being the most popular plant polysaccharides for bioplastic formation, starch-based films present low mechanical strength and have several drawbacks in their structural stability, caused by their stiffness or weakness due to their high hygroscopicity and very quick aging (Edhirej et al., 2016; Colivet & Carvalho, 2017; Zain, Kahar & Noriman, 2016). In addition, starch films have negligible antioxidant activity (Qin et al., 2019). Improving these characteristics is the biggest challenge in the production of biodegradable films that can be solved by blending with different biopolymers, yielding composite materials and bioactive additives in order to obtain active packaging (Santacruz, Rivadeneira & Castro 2015*; Sessini et al., 2016*; Manrich et al., 2017).

Nopal (*Opuntia ficus-indica*) is an economically important cactus plant that has several applications in different sectors of the industry widely used in the cosmetic and pharmaceutical industries because of its antioxidant, anticancer and anti-inflammatory properties (Espino-Díaz et al., 2010; Ayoub et al., 2014; Rodriguez-Gonzalez et al., 2014; Andreu et al., 2018; Bayar, Kriaa & Kammoun. 2016*;* Smida et al., 2017). Due to its mucilage and fiber content, nopal cladodes are also investigated as a source of bioactive compounds and an interesting candidate for the development of edible coatings and films (Ayadi et al., 2009; Sáenz, Sepúlveda & Matsuhiro, 2004; González Sandoval et al., 2019; Allegra et al., 2017; Scognamiglio et al., 2019; Luna-Sosa et al., 2020). Allegra et al., (2016) and Valle et al., (2005), state in their studies that nopal mucilage can be successfully applied to extend the useful life of highly perishable fruits, such as minimally processed strawberries and kiwi, with maintaining its natural characteristics. In addition, Espino-Díaz et al. (2010) states that mucilage extracted from nopal with the addition of a plasticizer has the ability to form films between pH values 4 and 8.

2 LITERATURE REVIEW

2.1 Nopal

Nopal (genus *Opuntia*, family Cactaceae), popularly known as forage crop in Brazil, is a plant originally from Mexico, but well adapted to other arid and semi-arid areas worldwide (Bayar, Kriaa & Kammoun. 2016; Contreras-Padilla et al., 2016). Due to the high genetic diversity, nopal is one of the most diverse and widely distributed genera in the Americas. Varieties (Figure 1) can be grown all over the world, in subtropical and cold temperature regions, being distributed in South America, North and South Africa, Middle Eastern countries and Mexico (Feugang et al., 2006) but it is also found in the southern United States, in Australia, Asia and Sicily (Ciriminna et al., 2019; Betatache et al., 2014; Saravanakumar et al., 2015). Brazil has the largest nopal plantation in the world, where the cultivated area occupies, including wide, around 900 000 ha (Ciriminna et al., 2019).

Figure 1. Different cultivars of *Opuntia ficus-indica* (FAO, 2013).

Currently, *Opuntia ficus-indica* is the main species of cactus distributed in the world for commercial production, as it produces edible fruits (prickly pear) and flat, fleshy stems known as cladodes (Ayoub et al., 2014; Santiago et al., 2018). Nopal cladode (~ 1.3 kg) is able to accommodate up to 93 wt.% of water. Thus, as livestock feed reserve plays an important role in animal nutrition and combating desertification process (Guevara, Suassuna & Felker, 2009). Nopal can be used in different forms, such as food, ornamentals, forages, as part of civil construction, cosmetic and medicinal and even incorporated in drinks (El-Mostafa et al., 2014). It is also used for pharmaceutical and cosmetic purposes, because it has been

shown to have antiviral, antidiabetic and anticancer properties (Feugang et al., 2006). This cactus has also been recognized to have significant coagulation activity in the actual wastewater treatment (Nharingo & Moyo, 2016). As human food, its cladodes are eaten peeled, fresh or cooked, but also as juices and in sauces (Santiago et al., 2018).

As well as the entire Cactaceae family, nopal cladode include mucilage, a complex highly branched polymeric structure rich in carbohydrates, which is produced in specific plant cells (Contreras-Padilla et al., 2016), and cellulosic fibers, that have a dietary value when fresh (Ciriminna et al., 2019). It is found to be low in protein (0.58 %) and lipid (0.12 %), moreover is abundant in dietary fiber (3.42%), with an elevated quantity of water-soluble fiber, including β-polysaccharides (Rocchetti et al., 2018).

One of the most interesting properties of nopal is its antioxidant capacity, that is, its ability to neutralize reactive oxygen species. This ability was assessed by eliminating the DPPH radical, one of the most widely used tests to estimate the anti-radical activity of antioxidants; this antioxidant activity is probably closely related to the presence of polyphenols in its composition (Bayar, Kriaa & Kammoun. 2016; Smida et al., 2017).

In the study conducted by Andreu et al., (2018) six nopal cultivars were investigated. All cultivars showed significant levels of phenolic compounds and antioxidant activity by the DPPH assay, in addition to citric and malic acid, glucose and fructose content. According to these authors, the total phenolic content of nopal turns this culture into an interesting material for the food industries.

Wit et al, (2019), analyzed the fruit pulp, peel, seeds and cladodes of nopal. The results showed that, in relation to the antioxidant action evaluated by the DPPH method, peel and cladodes were the ones that presented the greatest source of antioxidants, which can be explained by their specificities, since peel and cladodes have a protective function in plant. In addition, the phenolic and carotene content was higher in cladodes than in fruits, although

fruits have a greater capacity to chelate ferrous ions. The same authors highly recommended the inclusion of this cactus plant in the human diet.

Following this idea Ortiz et al., (2019) suggest that the presence of secondary metabolites in the nopal cladodes, its fibers content and phenolic compounds contributed to a greater intestinal fermentation of sows. The same study showed bacteriostatic activity of nopal cladodes, preventing the colonization of pathogenic bacteria in the intestines of these animals, which guaranteed intestinal integrity without affecting the quality of milk produced by them. Farias (2016) reported that the pre-treatment with nopal cladode mucilage presents an effective antioxidant protection, against gastric lesion induced by 50 % ethanol in Wistar rats. This author suggest that the powerful antioxidant effect of the nopal mucilage it is due to the presence of various aromatic compounds identified by NMR and ultra-performance liquid chromatography analysis (UPLC), including polyphenols and vitamins with high antioxidant capacity. Araújo et al., (2015), also identified the presence of superoxide dismutase (SOD) and catalase (CAT), enzymes that are considered to be an additional enzymatic defense against reactive oxygen species (EROS), in the nopal mucilage.

As seen, nopal mucilage has potential for application as an antiradical and as an industrial hydrocolloid, food additive, thickener, emulsifier and stabilizer (Bayar et al., 2017; Garti & Leser, 2001). Still in the food sector, it has been studied as an edible coating on fruits and as a source of bioactive compounds (Allegra et al., 2017; Ayadi et al., 2009; González Sandoval et al., 2019; Sáenz, Sepúlveda & Matsuhiro, 2004; Del-Valle et al., 2005). In addition, its high fiber content, beside very important in human nutrition, are also suitable, though, to act as a reinforcement of a polymer matrix, especially aiming at improving its tensile and bioactive properties (Scognamiglio et al., 2019). Thus, there is a call for new research to take advantage of the nopal cladodes respective constituents for food,

pharmaceutical and industrial applications, including its incorporation in polymeric blends for development of biodegradable films (Stintzing & Carle, 2005; Angulo-Bejarano et al., 2019).

2.2 Biodegradable films

Some alternatives to synthetic plastic have been introduced in the scientific and industrial fields, such as the replacement of fossil raw materials with renewable alternatives, focusing mainly on the development of more "sustainable" polymers for the development of biodegradable plastics. Biodegradable plastics, or films, are usually made with polymers extracted from plants and / or crustaceans (starch, pectin, polylactide (PLA), chitosan, etc.) (Zhu, Romain & Williams, 2016; Yang et al., 2017).

The term "biodegradable" is used for materials that can be degraded by living organisms, such as bacteria, yeasts, fungi and algae, through enzymatic action. As results of the degradation process are generated: CO_2, H_2O and biomass under aerobic conditions and hydrocarbon, methane and biomass under anaerobic conditions (Gross & Kalra, 2002). Also according to the same authors, the worldwide consumption of biodegradable polymers increased from 14 million kg in 1996 to around 68 million kg in 2001. Due to the benefits associated with the use of bio-based plastics, the development of new technologies is growing at an annual rate of up to 30% over the next decade. In line with current estimates, global production is expected to reach 3.5 million tonnes in 2020 (Shen, Worrell & Patel, 2010).

The target markets for biopolymers should include packaging materials, disposable nonwovens and agricultural tools, where synthetic plastics are used for applications with a short shelf life, such as film packaging, laminated paper, garbage bags, cotton buds and others (Avella et al., 2005).

Compared to synthetic plastic films, biodegradable films produced from a single source of biopolymers usually have poor mechanical and elongation properties and low water vapor permeability. To solve this problem, research has shown that the interaction between

two or more natural biopolymers produces high quality biodegradable films. The physical and mechanical properties of natural composite materials depend on the type and morphology of the fibers used, as well as their orientation in the polymeric matrix, since a good interaction guarantees good transfer of tension from the matrix to the filler material (Satyanarayana et al., 2009; Montaño-Leyva et al., 2013).

Edible films in addition to fulfill the basic function of packaging such as preserving color, texture and ideal humidity have the advantage of being able to be consumed with the food they contain. They can also provide food products better nutritional and sensory quality (Giosafatto et al., 2014). An edible coating or film can be defined as a thin layer of edible material formed on the surface of a food or present between the components of the food (preformed) with specific properties, which can be consumed together with the food (Bravin, Peressini & Sensidoni, 2006). They can also be developed with the incorporation of functional additives, such as vitamins, antimicrobial agents and antioxidants, providing extra protection against oxidative and microbial deterioration of food (Saha et al., 2016). These films are generally developed using lipids, polysaccharides, proteins and their compounds, used alone or in combination (Cavallaro et al., 2011; Sothornvit & Krochta, 2001).

According to the recently studied literature, different materials have been used for the production of edible films. Cutin, a high molecular weight biopolyester extracted from tomato peel, incorporated in a pectin matrix (Manrich et al., 2017), cassava starch, blended cassava starch with chitosan and blended potato starch with chitosan (Bergo et al., 2008; Santacruz, Rivadeneira & Castro 2015), gluten (Kumari et al., 2017), corn, potato and wheat starch (Basiak et al., 2017; Sessini et al., 2016) pea starch and guar gum (Saberi et al., 2017). The great advantage of study and develop biodegradable films is that they can be promisingly used in the packaging systems without the problem of disposability and environmental contamination.

2.2.1 Polymeric Blends

Most films made from a single natural polymer have unsatisfactory physical and mechanical properties, especially when compared to traditional synthetic materials (Montaño-Leyva et al., 2013). Therefore, a way to improve these materials is by mixing two or more biopolymers, these formulations are then called polymeric blends or composite materials.

Natural blends are obtained when two or more biopolymers (carbohydrates, proteins, lipids and fibers) obtained in the form of flour from raw materials of natural origin, are used in the formulation, The elaboration of these blends is favored by the natural thermodynamic compatibility of biopolymers, which prevent the appearance of phase separation (Grinberg & Tolstoguzov, 1997).

The films obtained from these blends will have their properties dependent on the interactions formed by the biopolymers, in addition to the distribution of these interactions within the film matrix, and the hydrophilic and hydrophobic interactions, as well as the concentration of each component used (Andrade-Mahecha; Tapia- Blácido and Menegalli, 2012; Tapia-Blácido et al., 2007).

Non-natural blends are those obtained from the mixture of polymeric materials in hot fluid form or through the dissolution of the components in the same solvent. The first technique is a method widely used in industry and the second is more usual for experiments on a smaller scale (Sionkowska, 2011).

Polymeric blends are advantageous, as they facilitate the adjustment of the properties of the polymers to the needs of use and application, since with them it is possible to control desired mechanical, chemical or barrier properties and at low cost, when compared to the synthesis of new polymers (Nascimento et al., 2013).

2.2.2 Film Forming Production

Regardless of the polymeric matrix used, the production of biodegradable film from natural resources requires the incorporation of a plasticizer into film formulations in order to improve the properties such as flexibility and handling, controlling the glass transition in a desirable manner (Andreuccetti, Carvalho & Grosso, 2009).

Plasticizers are generally small molecules such as polyols, glycerol and sorbitol, and organic acids, such as malic, tartaric and citric acid, which are inserted into a polymeric matrix to increase the free space between the chains, causing a decrease of intermolecular forces along the chains (Rodríguez et al., 2006). Glycerol, for example, is a natural plasticizer, the main by-product of biodiesel production and is widely added to the development of biodegradable and edible films (Jiménez et al., 2012).

To produce biodegradable films in laboratorial scale, the most widely used technique is the classical casting. This method consists in pouring a suspension on small containers (e.g., a Petri dish or acrylic plates). To control thickness of the resulting films, it is necessary to calculate the required mass of suspension poured on the plate, according to its own dimension. The suspension drying process takes place at room temperature or in ovens with forced air circulation at temperatures that varies between 30 and 40 °C, during 10–24 h to completely dry (de Moraes et al., 2013; Müller, Laurindo & Yamashita, 2009).

2.2.3 Cassava Starch

Cassava is widely grown in the tropical and subtropical regions of Asia, Africa and South America and due to the elevated starch content (20–40%) is the third most important source of plant-based calories, after rice and corn (Zhu, 2015). Brazil occupies a prominent position in the global production of cassava with 23.07 million tons produced in 2017, alongside only Nigeria which produced 41.53 million tons (FAO, 2019). Cassava plays an important role in the Brazilian economy, as the activities related to the cultivation of this

plant and its derivatives generate almost one million direct jobs in Brazil (Alves, Junior & De Campos, 2010). In 2018, the Gross Value of Agricultural Production (VBP) for cassava was R$ 9.78 billion, according to the Ministry of Agriculture, Livestock and Supply (BRASIL, 2019).

Starches are polymers naturally produced in the form of granules by certain plants, such as roots, tubers and cereals, in which they play the role of energy reserve (BeMiller & Whister, 2009). Its composition and shape vary according to the botanical source, as well as the genetic varieties of each species, but starches in general are usually composed of two polymers: amylose (approximately 20% of the polysaccharides found in the starch grain), which is formed by units of glucose joined by glycosidic bonds of type α-1,4, creating a linear chain (Figure 2, A), and amylopectin (Figure 2, B) which is formed by units of glucose joined in bonds of type α-1,4 and α-1,6, forming a branched structure (constitutes approximately 80% of the polysaccharides found in the starch grain) (Janssen & Mosciki, 2009; Vanier et al., 2017).

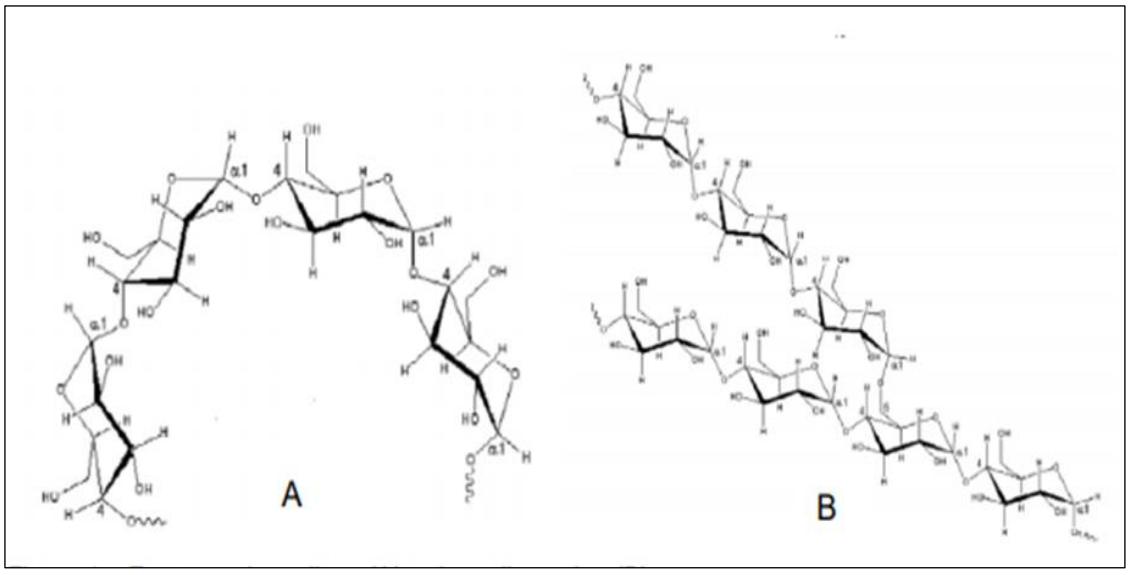

Figure 2. Structure of amylose (A) and amylopectin (B).
Source: (Souza, 2018).

Commercial extraction of starch uses grinding or grating techniques, followed by separation of fibers and suspension of starch in water, centrifugation, purification, dehydration and drying. However, some starches need adjustments in the extraction process. Thus, depending on the source, the method can be optimized to obtain better yields and higher quality of the final product (Agama-Acevedo et al., 2014).

2.2.3.1 Starch-Based Films

Starches are a viable alternative in the preparation of bioplastic materials, as it is inexpensive and renewable, being the predominant raw material for the production of biodegradable polymers. Among them, corn starch comes first, because it is the main source of starch produced in the world (64%), followed by sweet potato starch (13%) and cassava starch (11%) (De Souza; Ditchfield & Tadini, 2010; Luchese et al., 2018). Cassava starch still has the advantage of a lower retrogradation rate over starch from other sources, resulting in more stable materials over time (Mali et al., 2004).

The process of heating a suspension of starch in water to a certain temperature causes a process called gelatinization, where the starch absorbs the water molecules, causing their swelling, with consequent rupture of the granules and finally the loss of the original crystalline structure. Upon cooling, the gelatinization process is interrupted and the molecules that are in an amorphous state reorganize themselves, undergoing a recrystallization, more commonly known as retrogradation, however the polymers present undergo retrogradation at different speeds, quickly for the amylose molecules and more slowly and orderly for amylopectin molecules (Delcour et al., 2010). Retrogradation significantly influences the physical, mechanical and sensory properties of starch-based products (Vanier et al., 2017).

Starch has been used as a substitute for synthetic polymeric plastics. Polyvinyl alcohol (PVA), poly(lactic acid) (PLA) and polyester can also be mixed with starch (Bher, Auras & Schvezov, 2018; Saini, Arora & Kumar, 2016). The polymer obtained from the

mixture between starch and PVA is considered one of the most produced biodegradable plastic, being widely used in agriculture and in the manufacture of packaging (Liao & Wu, 2009).

Starch-based films are generally odorless, tasteless, colorless, non-toxic, and biologically absorbable and present as a good barrier to oxygen (Liu, Z. (2005). Even with all the properties listed, starch is considered a very difficult material to process; that's why starch-based films require the addition of some plasticizer during the development process. Due to its semi-crystalline nature, hydrophilicity and high solubility, starch-based films have undesirable feature, such as poor mechanical properties and low stability when compared to synthetic materials (Colivet & Carvalho, 2017; Zain, Kahar & Noriman, 2016). Starch-based films also have several drawbacks in their structural stability, which are caused by their stiffness or weakness due to their high hygroscopicity and very quick aging (Edhirej et al., 2016; Colivet & Carvalho, 2017; Zain, Kahar & Noriman, 2016).

In addition, starch films have negligible antioxidant activity (Qin et al., 2019). Improving these characteristics is the biggest challenge in the production of biodegradable films that can be solved by blending with different biopolymers, (kenaf, jute, sisal, coconut fiber, cane fiber, bagasse, etc. or eucalyptus or pine coniferous cellulose fibers) and bioactive additives in order to obtain active packaging (Santacruz, Rivadeneira & Castro 2015; Sessini et al., 2016; Manrich et al., 2017).

2.2.4 Nopal-Based Films

Nopal mucilage-based films have already been investigated. According to Espino-Díaz et al. (2010), nopal mucilage has the ability to form films between pH values 4 and 8. However, due to the rigidity and fragility of these films, the addition of plasticizer was necessary in order to improve the mechanical properties of the films. Also, Lira-Vargas et al., (2014), claim that films where nopal was a unique ingredient, regardless of the variety used,

presented a structure with many discontinuities and poor mechanical properties. In the study conducted by Gheribi et al., (2018), the development of nopal mucilage films as the main raw material was successful. The films were plasticized with four different types of plasticizer (glycerol, sorbitol, PEG 200 and PEG 400), obtaining different physical, thermal and mechanical characteristics that varied according to the plasticizer used.

The mucilage of nopal cladodes has already been successfully applied as an edible coating to prolong the shelf life of highly perishable fruits, with the maintenance of natural characteristics in kiwi slices (Allegra et al., 2016) and extending the shelf life of strawberries (Del-Valle et al., 2005). Fig fruits (*Ficus carica* L) treated with nopal coating showed higher visual quality scores and were still above the commercialization threshold, five days more than untreated fruits. In addition, there was a reduction in the development of microorganisms belonging to the Enterobacteriaceae family (Allegra et al., 2017). However, Ginestra et al., (2009) who studied the antimicrobial action of phytochemical fractions of nopal cladodes through the test of minimum inhibitory concentrations (MIC) did not demonstrate antimicrobial activity against the bacteria *Escherichia coli*, *Salmonella enterica*, *Pseudomonas putida*, *Listeria innocua*, *Lactococcus lactis* and *Staphylococcus aureus*, or against the yeast *Saccharomyces cerevisiae*. The lack of antimicrobial action can be understood most likely because of high content of polysaccharides that make up the nopal cladodes, which are present in the form of cellulose, hemicellulose and mucilage (Kuloyo et al., 2014). Ginestra et al., (2009) detected that 41% of the biomass of the dry cladodes was glucose.

Despite a limited number of published papers has already investigated the capacity of film-forming dispersion of nopal mucilage and reported its limitations as unique ingredient, the use of integral nopal cladode flour, including its fiber content, as reinforcement and bioactive agent of starch-based films has not been explored yet.

2.3 Reinforcement and Bioactive Agents

Starch-based films, as already discussed, have many disadvantages related to its sensitivity to water and poor mechanical properties (Da Róz et al., 2012; Gironès et al., 2012). To overcome these limitations, many approaches have been suggested in the literature; thus, one of the most effective methods for the development of biodegradable materials at low cost and with improved properties is through the incorporation of a reinforcement material, synthetic or natural, into the starch polymeric matrix (Aqlil et al., 2017; Sionkowska, 2011). As a natural alternative, the application of polymeric composites such as cellulose fibers and lignin as reinforcement material in starch-based films has grown (Zhang et al., 2018; 2020).

Whereas bioactive properties improvement can be solved by blending with bioactive and/ or antimicrobial additives in order to obtain active packaging (Santacruz, Rivadeneira & Castro 2015; Sessini et al., 2016; Manrich et al., 2017). The European Regulation EC No. 450/2009, classify active packaging as "Intentional incorporation of components that would serve as barrier for releasing or absorbing substances into or from the packaged food or the environment surrounding the food." (Jacob et al., 2020). Instead of mixing antimicrobial and antioxidant compounds directly into food, incorporating them into the films allows the functional effect to act directly on the food surface, where microbial growth and oxidative process are more likely to occur (de Araújo et al., 2015). These functional compounds act migrating from the packaging to food products or to the surrounding space; thus, being able to control the development of specific species of microorganisms and improve the oxidative stability of the packaged product (Costa et al., 2014; Petelinc et al., 2013; Piñeros-Hernandez et al., 2017; Suriyatem et al., 2018). Antimicrobial and antioxidant capacity of the film, however, will depend on the polarity and compatibility between the bioactive agent and the polymer matrix (Kaewprachu et al., 2018).

2.3.1 Lignin

Lignin is the second most abundant natural polymer after cellulose, but it is considered the most abundant aromatic biopolymer in nature. While cellulose and hemicellulose are energy-rich polysaccharides, lignin is a polyphenolic compound, with antioxidant activity and a likely energy reservoir for catalysis in plants (Margida, Lashermes & Moorhead, 2020; Shin et al., 2019).

The term lignin is derived from the Latin word "*lignum*" for wood. Lignin is a three-dimensional amorphous polymer that consists of methoxylated phenylpropane structures and is covalently linked to polysaccharides such as hemicellulose in the cell wall of plants (Norgren & Edlund, 2014). This compound can also be defined as the polymerized product of three major monomers: *p*-coumaryl alcohol, coniferyl alcohol and synaphyl alcohol (Figure 3), differing in methoxylation degree (Chakar & Ragauskas, 2004; Rencoret et al., 2015).

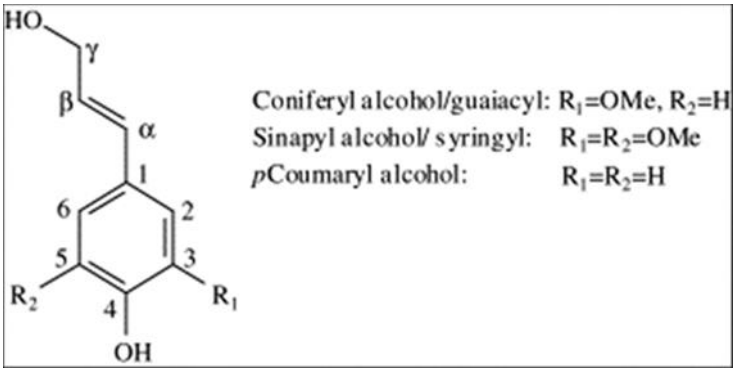

Figure 3. The three building blocks of lignin (Chakar & Ragauskas, 2004).

Lignins were divided into three main groups, according to the source, such as grass lignin, soft wood lignin and hard wood lignin. Although most lignins are separated or isolated from wood, they are usually called light wood lignin (MWL) or enzyme-released lignin (Roopan, 2017). The main role of lignin in the plant is to protect the cellulose polymer against hydrolytic attacks by pathogenic and saprophytic microorganisms (Ruiz-Dueñas &

Martínez, 2009). Its main monomers, such as p-coumaric, coniferyl and sinapyl alcohols, are toxic to bacteria due to the ability to selectively break the membranes of various microorganisms (Lou et al., 2012). However, it does not present cytotoxicity to human cells, being still considered a compound with antioxidant capacity (Jiang et al., 2015).

Klapiszewski et al., (2019), revealed in their study that a material composed of zinc oxide (ZnO) and lignin, in the proportion of 1:5 by weight, exhibited effective antimicrobial activity against Gram-positive bacteria (genera *Staphylococcus* and *Bacillus*), however, showed weak activity against *Salmonella and Enteritidis* (Gram-negative). The authors emphasize that the presence of ZnO nanoparticles in the material was essential and provided a synergistic effect to the antimicrobial action of lignin.

Yang et al., (2018), evaluated the antibacterial action of spherical lignin nanoparticles (LNP) and observed that LNP proved to be an efficient antibacterial agent against Gram-negative bacteria *Pseudomonas syringae, Xanthomonas axonopodis*, and *Xanthomonas arborícola*. According to the authors, the antimicrobial action of lignin occurs through lysis and subsequent penetration into the bacterial cell wall, where it reacts with ROS species, inducing oxidative stress, ATP depletion and decreasing the bacterial intracellular pH. It was also observed that the concentration of LNP is proportional to its antimicrobial activity, with LNP 8% by weight being more efficient than LNP 5%.

The bio-oil derived from the lignin pyrolysis also had proven antimicrobial activity, even at low concentration (0.3 mg / mL). Hossain et al., (2015), states that the lignin bio-oil demonstrated effective antimicrobial activity against the fungi *Pythium ultimum, Rhizoctonia solani* and *Sclerotinia sclerotiorum* and against the bacteria *Clavibacter michiganensis, Streptomyces* (both Gram-positive) *and Xanthomonas campestre* (Gram-negative).

Despite this, lignin is a product of low commercial value and has been considered a waste from biotechnology industries (Shin et al., 2019). Due to its vegetal origin, it can be declared as a natural and renewable raw material, being naturally degradable and biocompatible with most other natural polymers (Tuomela et al., 2000), making it an ideal precursor for the development of biodegradable materials. However, the reinforcing effect of lignin for the polymeric matrix depends intensively on the size of the particles and the strong interfacial bond with the matrix (Roopan, 2017).

2.3.1.1 Films with Lignin Added

The lignin network containing highly complex aromatic hetero polymers of phenylpropane units is thermally more stable than other polysaccharides extracted from plants (Poletto et al., 2012). In this sense, lignin has been an attractive material, since it acts by modifying the physical and chemical properties of thermoplastic starch (TPS). In addition, the incorporation of lignin in the polymeric starch matrix improves its mechanical properties, especially the tensile strength (Baumberger, 2002; de Miranda et al., 2015; Shi & Li, 2016; Spiridon, Teaca & Bodirlau, 2011). The abundance of carbonyl and carboxylic groups and the phenolic and aliphatic hydroxyl groups, functional groups that provide active sites for chemical modification, such as polarity adjustment, give lignin a good ability to establish a strong interfacial interaction with other polymers, making this biopolymer natural a suitable candidate to be incorporated into other polymeric matrices (Aqlil et al., 2017; Klein et al., 2019). Polymeric polyphenol lignin is bio-renewable and a natural antioxidant suitable for use in polymers, due to its lower sensitivity to high temperatures and higher molecular weight compared to low molecular weight natural antioxidants, generally proposed for the stabilization of polymeric materials, as is the case of α-tocopherol (Grande, Pessan & Carvalho, 2018).

Due to the hydrophilic nature inherent in starch, films from this matrix tend to absorb large amounts of water and, therefore, have a very hygroscopic behavior. Lignin, in turn, decreases the water content in equilibrium due to its aromatic fragments of hydrophobic character (Sarwono et al., 2018). Aqlil et al., (2017), reported that the addition of lignin within the starch matrix resulted in an important improvement in the performance of the resulting films. Lignin contributed to a significant reduction in the water solubility and final water content of this film. Young's modulus and tensile strength of the starch/lignin films increased and the elongation at break values decreased due to the strong interaction between the starch and lignin functional groups. Which indicated good compatibility between the hydrophobic lignin and the portions of hydrophilic starch. The presence of glycerol contributes to the miscibility of the two phases.

According to Sarwono et al., (2018), the good compatibility between the starch matrix and the lignin can be explained due to the amount of water-soluble phenolics present in the lignin, the hydrophilic character of these compounds helps in the interaction with the starch matrix, through the hydrogen bond.

The biocidal activity of PLA films or nanocomposites containing lignin nanoparticles was discussed by Spiridon et al., (2020). The results of the antimicrobial tests revealed an innovative capacity to inhibit the growth of Gram-negative bacteria, such as *Xanthomonas axonopodis* and *Xanthomonas arborícola*, considered bacterial pathogens dangerous for some plants. Klein et al. (2019), on the other hand, concluded that polyurethane coatings synthesized from demethylated Kraft lignins, proved to be efficient in the microbial reduction of Gram-positive bacteria *S. aureus.*

As seen, studies have been carried out regarding the incorporation of lignin in several matrices for the manufacture of polymeric products with microbial growth control and with antioxidant capacity, providing added value in the different fields (Spiridon et al., 2020;

Spiridon, Anghel & Bele, 2015). The migration values of these compounds for the studied systems are well below the general legislative limits of migration, suggesting that lignin can be considered suitable as fillers in polymeric matrices and, consequently, for application in the formulations proposed for food packaging (Agustin-Salazar et al., 2018; Spiridon et al., 2020; Yang et al., 2016). The main applications for starch-lignin biocomposites would be packages for single or short-term use, as naturally biodegradable alternatives to conventional synthetic polymers, thus presenting themselves with great potential to be used as fresh food packaging (Aqlil et al., 2017).

2.3.2 Propolis

Propolis is a bee product of worldwide interest due to its biological properties, being produced by bees from exudates collected from the buds and flowers of the diverse flora in the vicinity of the hive. Propolis is formed when these botanical exudates are chewed by bees (*Apis mellifera*), mixed with salivary enzymes such as β-glycosidase and incorporated with beeswax (Silva et al. 2012). Propolis is for the purpose of sealing holes in the hive, which provides protection against intruders and prevents putrefaction and infections from spreading throughout the colony (Oliveira et al., 2015; Suleman et al., 2015). The variability in its composition is due to the botanical origin of vegetable resins harvested by bees, as well as the climate and geographical location of the hives (Toreti et al., 2013).

The main types of propolis studied are called green, red and poplar propolis and are classified according to their chemical composition (Zancanela et al., 2019). For example, Brazilian green propolis (or simply green propolis) is a type of propolis found in southeastern Brazil, where bees consistently collect unexpanded leaves of *Baccharis dracunculifolia* as a raw material for the preparation of propolis (Kumazawa et al., 2003; Saito et al. , 2020). On the other hand, red propolis may have Cuban or Brazilian origins, the Brazilian found in the

Northeast region, which is prepared with exudates collected from Dalbergia ecastophyllum (Piccinelli et al., 2011). Propolis from Europe (or “poplar” propolis) is found in Europe and other temperate zones, where bees collect sprouts of Populus species as raw material for the preparation of this type of propolis (Bankova, 2005).

Among tropical countries, Brazil has the greatest chemical diversity of types of propolis. Brazilian propolis is a highly valued bee product and of enormous commercial importance, which is divided into two most common varieties: green and brown propolis (Salatino et al. 2011). Brazilian green propolis is the most abundantly produced and consumed, internally or externally (Righi, Negri & Salatino, 2013). There are more than 300 compounds in propolis, however, it can be said that green propolis is rich in pre-phenylated phenylpropanoids, triterpenoids, chlorogenic and benzoic acids, while brown propolis contains mainly flavonoids and terpenes (Nina et al., 2016; Salatino et al. 2011). According to Nina et al., (2016), the antioxidant activity of propolis can be explained by its composition, and the propolis with the most active antioxidant activity were those with the highest content of phenolics and flavonoids, while propolis containing mainly non-triterpenes had antioxidant activity.

Propolis contains phenolic compounds in large quantities, having shown considerably high antimicrobial and antioxidant activities, when compared to honey (Meda et al., 2005; Socha et al., 2015). However, the antibacterial action of propolis can be considered bacteriostatic or bactericidal, depending mainly on the concentration used (Righi, Negri & Salatino, (2013). Several researchers have obtained higher propolis antimicrobial activities against Gram-positive bacteria than against Gram-negative bacteria (Campos et al., 2014, Silici & Kutluca, 2005).

In their study Osés et al., (2016), observed that propolis showed antimicrobial activity against the microorganisms *Lactobacillus plantarum, Penicillium nordicum*, while

Penicillium expansum and *Pseudomonas aeruginosa* were resistant. Campos et al., (2014) evaluated the antimicrobial property of the ethanol extract of propolis produced by *Melipona orbignyi* bees, where activity against the gram-positive bacteria *Staphylococcus aureus* and the fungus *Candida albicans* was observed. However, it was not effective against gram-negative bacteria E. coli. Lu, Chen & Chou, (2005), also observed that the ethanolic extract of propolis samples have an antibacterial action against *S. aureus*, a pathogen frequently reported to produce food poisoning all over the world. Showing that propolis may hold potential as an antimicrobial preservative. However, pH and temperature are factors that should be concerned during propolis application. The antimicrobial activity of propolis can be attributed to the presence of phenolic compounds that inhibit bacterial growth via inhibition of bacterial RNA polymerase and disruption of bacterial cell membrane and cytoplasm (Costa et al., 2014; Petelinc et al., 2013).

A hydroethanolic extract is the most common formulation of propolis found on the market (Funari et al., 2016). However, due to the inadequacy of the exposure of certain individuals to ethanol, such as ex-alcoholics and children, other extracts of aqueous formulation and with propylene glycol are also eventually marketed, but on a smaller scale (Funari et al., 2019). Aqueous formulations are alcohol-free, but retain the characteristic flavor and smell of propolis, in addition to having a lower cost compared to hydroalcoholic extracts (Passos et al., 2016; Sforcin, 2007). Recently, extraction methods have combined ecological and high-yielding solvents in the isolation of bioactive compounds. Green extraction, as it is so known, uses aqueous solutions and excludes organic solvents or high energy consumption methods (Vasilaki et al., 2019).

The antioxidant effect of aqueous propolis extract was also confirmed in an in vivo test, carried out by Khayyal, Abdel-Naby & El-Ghazaly, (2019), when assessing the number of intestinal lesions caused by oxidative stress when subjected to the radiation

process, the protective effect of the aqueous propolis extract is possibly a result of its efficiency in eliminating free radicals. Result similar to that reported by El-Ghazaly, Rashed & Khayyal, (2011). The activity of these extracts has been attributed mainly to their content of flavonoids and derivatives of caffeic acid (Khayyal, El-Hazek & El-Ghazaly, 2015). It was also observed that aqueous and ethanolic extracts showed inhibitory effect against the species of fungus *C. albicans*, however the ethanolic extract was more effective compared to the aqueous extract (Sayyadi et al., 2020).

2.3.2.1. Films with Propolis Added

The addition of propolis ethanolic extract as an antimicrobial agent in biodegradable films has been extensively studied, due to its good incorporation into polymeric matrices and effective capacity against some microorganisms (Costa et al., 2014; Pastor et al., 2010; Shavisi et al., 2017; Suriyatem et al., 2018).

Pastor et al., (2010) observed that the incorporation of ethanol extract of propolis in films of hydroxypropyl methylcellulose (HPMC), a common hydrocolloid used for the formulation of biodegradable films, did not produce a negative effect on the mechanical characteristics in the matrix. However, the presence of propolis significantly reduced the population of the fungus *Aspergillus niger*. However, it does not demonstrate an effective antimicrobial activity against *Penicillium italicum*.

Rice starch/carboxymethyl chitosan composed films enriched with propolis extract (5 and 10%) showed antimicrobial activity against *Staphylococcus aureus* and *Bacillus cereus*. However, no zone of inhibition for E. coli was observed in all films. This study revealed that the active films inhibited Gram-positive bacteria, but not Gram-negative bacteria in the investigated propolis levels (Suriyatem et al., 2018). What is in agreement with other studies already carried out (Campos et al., 2014; Silici & Kutluca, 2005; Siripatrawan, Vitchayakitti & Sanguandeekul, 2013). A structural difference between Gram-positive and

Gram-negative bacteria cells may be an explanation for this behavior. As Gram-negative bacteria, in addition to the cell membrane, they have an additional outer layer composed of lipopolysaccharides (endotoxins), lipoproteins and phospholipids that are impervious to most foreign molecules (Silva et al., 2012; Suriyatem et al., 2018).

De Araújo et al., (2015) also observed that the addition of propolis extract (1%) in cassava starch film showed activity against Escherichia Coli and against *S. Aureus*, as expected. Costa et al., (2014), evaluated the addition of red propolis to a cassava starch film. The addition of propolis extract alone did not affect the mechanical properties of the films: similar results were obtained for the control films and in the films enriched with propolis, but it showed an antimicrobial effect against coagulase-positive staphylococci, which include *S. aureus*, S. *intermedius* and *S. hyicus.*

3. OBJECTIVES

3.1 General Objectives

With the perspective of reducing the environmental impact caused by synthetic plastics and in order to address nopal cladodes in a more noble and economically feasible application in Brazil, this research aims to process nopal cladodes in the form of flour and to use it as a biopolymer in the development of cassava starch-based films, also to envaluate the influence of the alkaline treatment applied to the nopal flour on the film properties as well as to compare the functional efficacy of the nopal flour to propolis extract and lignin addition.

3.2 Specific Objectives

- Process nopal flour, through grinding and sieving the cladodes (*Opuntia ficus-indica*);
- Evaluate the influence of particle size on the chemical properties of nopal flours;
- Characterize the surface of the flour granules by SEM;

- Elucidate the chemical structure of the nopal flour;

- Treat nopal flour by adjusting the pH of the solution by using sodium hydroxide solution;

- Develop biodegradable films by incorporating nopal flour into a polysaccharide matrix of cassava starch;

- Characterize the physical, mechanical properties and the antioxidant capacity of the obtained starch based-films;

- Develop starch/nopal, starch/lignin and starch/propolis-based films;

- Evaluate the effect of nopal, lignin and propolis addition on physical and mechanical properties, as well as to compare its influence on the bioactive and antimicrobial properties of the starch based-films.

- Evaluate the effect of alkaline treatment on nopal flour on the properties of starch/nopal-based films reinforced with lignin and propolis extract.

4 FINAL CONSIDERATIONS

The key issues addressed to this thesis studied were: a) Is it possible to produce a bio-based film using nopal cladode whole flour (NC) itself? b) Could be possible its incorporation in another polymer matrix, such as cassava starch? c) Does the alkaline treatment under different pH values of the cladodes flour have an influence in the final film properties? d) Is it possible to compare the effect of the addition of the nopal flour to the addition of propolis or lignin on the bioactive properties of cassava starch based-films? How do all these components behave together?

We observed that due the fiber content it was not possible to form film using the nopal cladode whole flour by casting method. Also, the results achieved showed that nopal addition afforded films with yellowish color and the alkaline treatment applied to the nopal flour had a greater influence in the increase of the hydrophobicity of the film surfaces and affected more the water resistance of the films than the addition of lignin and propolis extract in the tested concentrations. The films did not show inhibition zone against all of the four microorganisms tested, although nopal addition contributed most with the total phenolic compounds content, and antioxidant activity by ABTS and DPPH, and afforded films with high antioxidant activity. Thus, cassava starch/nopal film is suitable for incorporation into active packaging systems reducing the oxidation process and extended the maintenance of quality of high oxidative food during storage. In addition these data in their entirety suggest that this polymer blend has the potential to associate satisfactory mechanical and functional characteristics.

For this, as a suggestion for new research would be the application of films in dry foods, such as nuts in general, as well as in cosmetics with a high oxidation rate. However, in order to obtain a film with antimicrobial capacity, it will be necessary to incorporate a biocidal agent more efficient and broad spectrum.

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APPENDIX A - PAPER 1

(SUBMITTED TO FOOD PACKAGING AND SHELF LIFE)

NOPAL CLADODE (*OPUNTIA FICUS-INDICA*) FLOUR: PRODUCTION, CHARACTERIZATION, AND EVALUATION FOR PRODUCING EDIBLE FILM

Abstract

We aimed to prepare and to characterize nopal cladode whole flour (NC) and to evaluate its use in edible film production. NC was obtained by milling and sieving through 45-, 80-, and 120-mesh sieves. The chemical composition, physical properties (analyzed by SEM, XRD, and FT-IR), and total phenolics and antioxidant activity (analyzed by the DPPH and ABTS methods) of the resulting flours (NC-45, NC-80, and NC-120) were characterized. FTIR spectra of NCs showed bands related to proteins, fiber, lipid, sugars, and pectin. XRD pattern of NCs showed presence of crystallites related to calcium salts, and peaks of cellulose type I and hemicellulose. NC-120 presented the smallest average particle size (166 μm), the lowest carbohydrate content (72%), and the highest lipid content (2.9%) and antioxidant activity, so it was chosen to prepare films by casting; glycerol was added as plasticizer. Because NC-120 alone failed to provide films, this flour was added to cassava starch, to give the starch/NC-120 film. Although the latter film displayed poorer mechanical properties than the cassava starch film, it presented better barrier to water vapor, important phenolics compound content (13.67 ± 0.5 mg of GAE/g of film) and antioxidant activity measured by DPPH (88.43 ± 4.19%) and ABTS (7.64 ± 0.60 μM Trolox/g of film). Thus, NC addition to cassava starch yielded bioactive starch-based films for use as packaging material to prevent food oxidation.

Keywords: nopal, cassava starch, bioactive activity, antioxidant activity, edible film.

1. Introduction

Nopal *(Opuntia ficus-indica)* belongs to the family *Cactaceae* and consists of four main parts: cladodes (succulent stems), flower, fruit, and seeds. This plant adapts to the environment easily and occurs in geographic areas and habitats that include the Mediterranean basin, the Middle East, South Africa, Australia, South America, Central America, North America, and India (Hernández-Hernández et al., 2011; Loretta et al., 2019).

Cladodes, also known as pads or nopales, contain mucilage and cellulosic fibers and are composed of water (80–95%) as well as small amounts of carbohydrates (3–7%), fibers (1–2%), and proteins (0.5–1%) (Zhao et al., 2017). The sugar moiety includes mucilaginous components consisting of polymers like (1-4)-linked β-D-galacturonic acid chains and R(1-2)-linked L-rhamnose residues (Trachtenberg S, Mayer, 1981; Lee et al., 2003). Cladodes also represent a source of phytochemicals, such as phenolic acids (aromadendrin, taxifolin or dihydroquercetin, isorhamnetin, vitexin, kaempferol, quercetin, betalains, betacyanins, rutin, and isorhamnetin) and flavonoids, and they contain citric and malic acids and some glucose and fructose (Blando et al., 2019; Ginestra et al., 2009; Osuna-Martínez, Reyes-Esparza, Rodríguez-Fragoso, 2014; Andreu et al., 2018; Wit et al., 2019). For this reason, some authors recommend that cladodes be included in the diet to improve the gastrointestinal health of suckling sows (Ortiz et al., 2019).

Mucilages and pectins have been extracted from the cladodes and peels of prickly pears (nopal fruits) and have been applied as thickening, gelling, emulsifying, and film-forming agents in food, cosmetic, and pharmaceutical and personal-care products. Some authors have suggested that nopal mucilage can be used as bioplastic and biomaterial for food packaging and biomedicine (Scognamiglio et al., 2019; Sáenz et al., 2004; Ayadi et al., 2009). Mucilage extracted from cladodes has also been employed as coating to increase the shelf life of perishable fruit like kiwi fruit and ‘Dottato’ fig fruit (Allegra et al., 2016 and 2017).

However, mucilage films have elevated water vapor permeability even in the absence of a plasticizer (Espino-Díaz et al., 2010; Gheribi et al., 2018). In turn, cladode cellulosic fibers have been applied as reinforcing agent in thermoplastic starch based on potato starch and glycerol (Scognamiglio et al., 2019). Nevertheless, research into edible films containing nopal cladode whole flour, and not only its individual components, has never been conducted.

The pollution issues caused by plastics have sparked interest in developing biodegradable materials that can replace synthetic plastics (Sessini et al., 2016; Parra et al., 2004; Farhan and Hani, 2017; Zhu et al., 2016). Among natural polymers, starch has been the most investigated for edible film production: it is naturally abundant, biocompatible, biodegradable, and non-toxic (Yuliana, et al., 2012; Gross and Kalra, 2002; Afolabi, et al., 2012). However, starch films have poor mechanical properties and absorb water easily (Dai, Zhang and Cheng, 2020). Furthermore, few types of starch display bioactive properties. Examples of starch with this kind of property include starch isolated from babassu mesocarp, starch extracted from annatto, and starch obtained from turmeric residue, which exhibit antioxidant activity (Maniglia et al., 2017 and 2019; Silveira & Tapia-Blácido, 2018).

In this study, we aimed to produce and to characterize the physicochemical and antioxidant properties of nopal cladode whole flour and to evaluate its use for edible film production.

2. Methodology

2.1 Materials

Nopal cladodes (*Opuntia ficus-indica)* were freshly harvested in the Northeastern region of Brazil (3.7327° S, 38.5270° W) at a plantation belonging to the Animal Science Department of the Federal University of Ceará (Ceará, Brazil). Cassava starch (with 18 wt.% amylose, 82 wt.% amylopectin, and 1×10^8 g/mol molecular weight) was purchased at a market located in Ribeirão Preto (São Paulo, Brazil). Glycerol and the radical scavenger's

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) and ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) were supplied by Sigma-Aldrich (São Paulo, Brazil).

2.2 Preparation of nopal cladode flour

Nopal cladodes (*Opuntia ficus-indica)* were washed, cut into cubes, and dried in an oven with forced air circulation (60 °C/12 h) (Quimis, Q314M292, Brazil). Then, the dried nopal cladodes were grounded and sieved through 45-, 80-, and 120-mesh sieves, which afforded the nopal cladode flours NC-45, NC-80, and NC-120, respectively.

2.3 Chemical composition of nopal cladode flour

The moisture (AOAC 920.151, 1997), ash (AOAC 923.03, 1997), lipid (Bligh & Dyer, 1959), and protein (AOAC 926.86, 2005) contents of NC-45, NC-80, and NC-120 were analyzed, in triplicate.

2.4 Total phenolic compound quantification and antioxidant activity of nopal cladode flour

To determine the total phenolic compound content and the antioxidant activity of NC-45, NC-80, and NC-120 by the DPPH and ABTS methods, first an extract of the flour was prepared with deionized water. Then, the total phenolic compound content was determined according to Larrauri et al. (1997), with some modifications. To this end, 0.5 g (or 5% w/w) of NC-45, NC-80, or NC-120 was slightly stirred with 9.5 g of distilled water and heated at 40 °C for 10 min. The resulting extract was filtered and stored. Later, 30 µL of the extract was mixed with 470 µL of distilled water, 500 µL of Folin-Ciocalteau solution (1:3), 1 mL of sodium carbonate (20%), and 1 mL of distilled water, in this sequence. Then, the mixture was incubated in the dark for 30 min. After that, the absorbance was measured at 700 nm. The result is expressed as mg of gallic acid equivalent per g of flour.

The antioxidant activity NC-45, NC-80, and NC-120 was determined by the DPPH (Martins et al., 2012) and ABTS methods (Rufino et al., 2007). The NC-45, NC-80, or NC-120 extract was obtained by stirring 100 mg of NC-45, NC-80, or NC-120 with 2 mL of distilled

water, which was followed by stirring at 25 °C for 3 h. Then, 500 μL of the supernatant was mixed and reacted with 2 mL of the DPPH radical (0.06 mmol/L) and incubated at 25 °C in the dark for 30 min. After that, the absorbance was read at 517 nm with a spectrophotometer (HP Hewlett Packard 8453, USA). The control consisted of 500 μL of methanol without flour. All the analyses were performed in triplicate.

2.5 Particle size distribution of nopal cladode flour

The particle size distribution of NC-45, NC80, and NC-120 was determined by laser diffraction with static light scattering (SLS). A BeckmanCoulter laser diffractometer (BeckmanCoulter, model LS 13320, USA) equipped with a tornado module was employed.

2.6 X-ray diffraction analysis of nopal cladode flour

X-ray diffraction analysis of NC-45, NC-80, and NC-120 was carried out on a Siemens X-ray diffractometer (Siemens, model D5005, Baden-Württemberg, Germany) operating at 40 kV and 30 mA with Kα copper radiation and monochromatic filter, at room temperature. The data were collected from 2 to 50 °C on a 2θ scale, at a rate of 0.02° / s. The crystallinity index (%) of the flours was quantitatively estimated as the ratio of the crystalline area of the individual peaks by the total area of the total diffractogram area. The Nara and Komiya (1983) method was followed, and the software Origin 8.1 was used to plot the graphs (OriginPro Corporation, Massachusetts, USA).

2.7 FT-IR analysis of nopal cladode flour

Fourier Transform Infrared (FT-IR) analysis of NC-45, NC-80, and NC-120 was accomplished to determine their functional groups. The spectra were recorded from 500 to 4000 cm^{-1} on a Shimadzu FT-IR spectrometer (Shimadzu, model IR Prestige-21, Kyoto, Japan) with Fourier transform; the nominal resolution was 2 cm^{-1}.

2.8 Evaluation of nopal cladode flour for film production

NC-120 was pretreated before it was used to prepare the film. To this end, 5 g of NC-120 was dispersed in 95 g of distilled water and homogenized with a homogenizer at 10,000 rpm and room temperature for 4 min (Tecnal, TE-102 Turratec). The dispersion was kept in dark containers at room temperature (~25 °C) for 12 h. Then, the dispersion was filtered (150-mesh sieve), to separate the largest particles that could impair the formation of a homogenous film. The filtered solids were separated, and the supernatant containing 2.75 g of NC-120 was used to prepare the composite films.

2.8.1 Preliminary test of nopal cladode flour film production

A film based on NC-120 only was initially prepared. Briefly, 100 g of filmogenic solution was obtained by using 5% (w/w) NC-120 and 1% (w/w) glycerol, as plasticizer. The suspension was heated at 85 ± 1 °C until a semi-viscous solution emerged. The dispersion was cast onto an acrylic plate (0.155 g per cm^2) and dried in an oven (BOD, Marconi) at 32 °C for 10 h.

2.8.2 Preparation of starch/NC-120 film

Cassava starch was added as polymer matrix. Thus, a blend of NC-120 and cassava starch was prepared and designated starch/NC-120. Specifically, 100 g of filmogenic solution was achieved by incorporating 4 g of cassava starch into 95 g of the previously treated NC-120; 1% (w/w) glycerol was used as plasticizer. The suspension was heated at 85 ± 1 °C until a semi-viscous solution emerged. The dispersion was cast onto an acrylic plate (0.155 g per cm^2) and dried in an oven (BOD, Marconi) at 32 °C for 10 h. The dried film was conditioned in a desiccator at 25 °C and 53% relative humidity (RH) for 48 h before being analyzed. Cassava starch film without added nopal cladode flour was also produced as control.

2.9 Film characterization

The average thickness of the preconditioned films (starch/NC-120 and cassava starch films; 58% RH, 25 °C) was calculated from three measurements at random positions; a Zaas-Precision flat tip digital micrometer (1-μm resolution) was employed.

Mechanical tensile strength was measured with a TA Texturometer (TA Instrument, model TX Plus) according to ASTM D882-09 (ASTM, 2002). The films were cut into 2.54 cm-wide and 10 cm-long strips, and five samples of each formulation were analyzed. The initial grip separation and the crosshead speed were set at 80 mm and 1.0 mm s^{-1}, respectively. Tensile strength (force/initial cross-sectional area) and elongation at break were directly determined from the stress × strain curves by using the software Texture Expert V.1.22 (SMS, Surrey, U.K.). Young's modulus was calculated as the slope of the initial linear portion of this curve.

Moisture content was determined gravimetrically. The films were dried in an oven at 105 ^{o}C for 24 h, and their moisture content was calculated according to ASTM D644-99 (ASTM 1999).

Solubility in water was evaluated as described by Tapia-Blacido, Sobral, and Menegalli (2005): three film discs with 20-mm diameter were weighed, immersed in 50 mL of water, and stirred at 25 ± 2 °C for 24 h. The non-solubilized fractions were separated by filtration and dried at 50 °C until constant weight, and the percentages of dry matter were calculated.

The water vapor permeability (WVP) test was accomplished by using a modified ASTM-Standard-method-E96-96M (ASTM, 2014) as described by Martelli-Tosi et al. (2017). The films were sealed over a permeation cell containing silica gel and placed in desiccators containing distilled water until steady-state conditions were reached. The cell was weighed every 1 h for 9 h. WVP was calculated as WVP = w.x/t.A.P, where "x" was the average thickness of the films, "A" was the permeation area (0.00196 m^2), and "P" was the difference between the partial pressure of the atmosphere over silica gel and over pure water (3.168 kPa,

at 25°C); the term w/t was calculated by linear regression of the data of weight gain as a function of time.

Wettability was analyzed by measuring the contact angle with OCA-20 Dataphysics (Germany). The water contact angle is defined as the angle between the water drop and the film surface (Ojagh et al., 2010) and is expressed in degrees.

Total phenolic compound content and antioxidant activity were also determined by the DPPH and ABTS methods according to the same methodologies that were used for NC-45, NC-80, and NC-120. All the analyses were conducted in triplicate.

2.10 Scanning electron microscopy (SEM) of nopal cladode flour and films

The morphology of NC-45, NC-80, and NC-120 flour and of the starch/NC-120 and cassava starch films was analyzed by Scanning Electron Microscopy (ZEISS scanning electron microscope, model EVO-50). Each sample was covered with gold by sputtering (Bal-Tec SCD 050) and accelerated under 20 kV.

2.11 Statistical analysis

The data were subjected to one-way ANOVA analysis with 95% confidence interval by using the software *Statistica 7.0*. Tukey's test was performed to determine significant differences ($p < 0.05$) in the properties of NC-45, NC-80, and NC-120 flours. This test was also carried out to evaluate differences between the starch/NC-120 and cassava starch (control) films.

3. Results and discussion

3.1. Chemical composition of nopal cladode flour

Table 1 summarizes the chemical composition of NC-45, NC-80, and NC-120. The flours did not present significant difference for moisture and ash contents. However, NC-120 exhibited statistically less carbohydrate and higher lipid content than NC-45 and NC-80.

The carbohydrates included fibers and mucilage; the latter comprised sugars such as arabinose, galactose, rhamnose, and xylose (Lee et al., 2003). Compared to NC-45, NC-80, and NC-120, El-Safy (2013) reported lower content of other carbohydrates (46%) and similar lipid content (2.2%) to NC-120 in nopal cladode flour sieved through 60-mesh sieves.

The protein and ash contents of NC-45, NC-80, and NC-120 resembled the protein and ash contents in dry cladodes reported by Du Toit, De Wit, and Hugo (2018) (~3.2% protein and ~18% ash) and Du Toit et al. (2019) (~3.4% protein), but they were lower than the contents reported by Ayadi et al. (2009) (~8.8% protein and ~24% ash) and Yang et al. (2015) (~7.4% proteins and 23.7% ash). Furthermore, NC-45, NC-80, and NC-120 had higher protein content than the dehydrated cladodes produced by Espino-Díaz et al. (2010) by freeze-drying (1.04%), but lower protein content than the nopal cladode flour produced by El-Safy (2013) (7.3%). The differences in the chemical composition of NC-45, NC-80, and NC-120 and of materials obtained by other authors can be attributed to the nopal cultivation conditions including soil, climate, and growing region.

Table 1. Chemical composition of the nopal cladode flours NC-45, NC-80, and NC120

Flour	Moisture (%)*	Ash (%)	Lipids (%)	Protein (%)	Carbohydrate (%)
NC-45	11.1 ± 1.6^{a}	20.4 ± 1.1^{a}	0.80 ± 0.19^{b}	3.31 ± 0.42^{a}	75.4 ± 0.48^{a}
NC-80	11.3 ± 0.2^{a}	21.7 ± 0.6^{a}	0.84 ± 0.24^{b}	1.9 ± 0.12^{b}	75.5 ± 0.62^{a}
NC-120	10.9 ± 0.8^{a}	21.4 ± 0.2^{a}	2.9 ± 0.37^{a}	3.3 ± 0.10^{a}	72.4 ± 0.60^{b}

Values with different lowercase letters in the same column are significantly different according to Tukey's test ($p < 0.05$).
*Values expressed on dry basis. *Moisture expressed on humidity basis.
Carbohydrate (obtained by difference) = 100 – (Ashes + Lipid + Protein).

3.2. Total phenolic content and antioxidant activity of nopal cladode flour

Table 2 lists the total phenolic compound content and antioxidant activity of NC-45, NC-80, and NC-120 determined by the DPPH and ABTS methods. According to Tukey's

test ($p > 0.05$), the flours showed no significant difference in terms of total phenolic content. Therefore, milling and sieving did not affect the total phenolic content of nopal cladodes.

NC-45, NC-80, and NC-120 had statistically antioxidant activity difference as analyzed by the DPPH and ABTS assays. Despite all the flours showed high antioxidant activity, as revealed by the DPPH (from 86 to 88%) and ABTS (from 18.79 to 20.93 µM Trolox/g of NC flour), NC-120 presented statistically highest antioxidant activity by ABTS assay. The nopal cladodes of six cultivars analyzed by Wit et al. (2019) showed similar antioxidant activity by the DPPH assay—from 83 to 95%. Meanwhile, NC-45, NC-80, and NC-120 showed higher antioxidant activity measured by ABTS than the value reported by Monter-Arciniega et al. (2019) for cladodes (14.85 µM Trolox g^{-1} dry basis). Therefore, milling and sieving used in this work did not negatively impact on the radical scavenging ability of nopal cladodes.

Table 2. Determination of total phenolic compound content and antioxidant activity in NC-45, NC-80, and NC-120.

Flour	Total Phenolics*	DPPH (%)	ABTS*
NC-45	12.33 ± 0.07^{a}	86.85 ± 0.36^{b}	18.79 ± 0.48^{b}
NC-80	10.56 ± 0.23^{a}	88.02 ± 1.01^{a}	18.97 ± 0.46^{b}
NC-120	13.49 ± 0.45^{a}	87.49 ± 0.66^{ab}	20.93 ± 0.20^{a}

Values with different letters in the same column are significantly different according to Tukey's test ($p < 0.05$).
*Total phenolics are expressed as mg GAE/g of NC flour.
*ABTS is expressed as µM Trolox/g of NC flour

3.3 Particle size distribution of nopal cladode flour

On the basis of Figure 1, NC45 and NC120 had unimodal particle size distribution, whereas NC-80 had polymodal particle size distribution. The main peak corresponding to the average particle size of NC45 and NC120 emerged at 491 and 166 µm, respectively. Meanwhile, NC-80 showed a mean average particle size peak at 213 µm and a

smaller peak at 493 µm. The values of d90 for NC-45, NC-80, and NC-120 revealed that NC-45 presented a particle size of 864 µm, and that sieving reduced the particle size at d90 to 600 and 373 µm for NC-80 and NC-120, respectively. According to Rodriguez et al. (2019), granulometry can affect the distribution of macronutrients and functional components, so particle size is directly related to the strength of brittle materials (Pino & Baudet, 2015). Grinding and sieving help to achieve powder with better properties, including water and oil holding capacity, and improve the release of bioactive compounds during processing (Goh, Heng & Liew, 2018, Nabil et al., 2020). Here, we observed that size reduction by milling and sieving affected the lipid and carbohydrate contents of the flour with the smallest particle size (NC-120). Indeed, NC-120 had statistically higher lipid and lower carbohydrate contents than NC-45 and NC-80 (Table 1). Thus, NC-120 could be an interesting raw material to produce edible films because its high lipid content could contribute to the hydrophobic character of the film. Moreover, this flour displayed the greatest antioxidant activity measured by ABTS (Table 2), which indicated that NC-120 retained hydrophobic compounds with antioxidant activity.

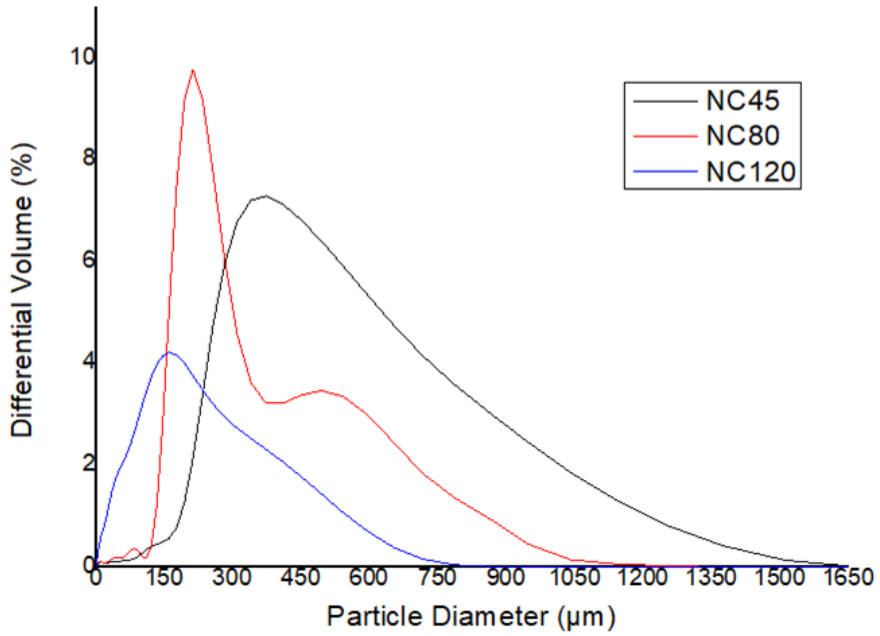

Figure 1. Particle size distribution of the nopal cladodes flours NC-45, NC-80, and NC-120.

3.4. Visual aspect and microstructure of nopal cladode flour by SEM

Figure 2 shows the SEM micrographs of NC-45, NC-80, and NC-120 at a scale of 100 μm and magnification of x100. The micrographs confirmed that NC-45, NC-80, and NC-120 had different particle size. The micrographs also evidenced two main structures in the flours: fibers and mucilage. The fibers presented long structures, which were greater in NC-45. The mucilage displayed porous structure and polygonal shape as also observed by Du Toit et al. (2019).

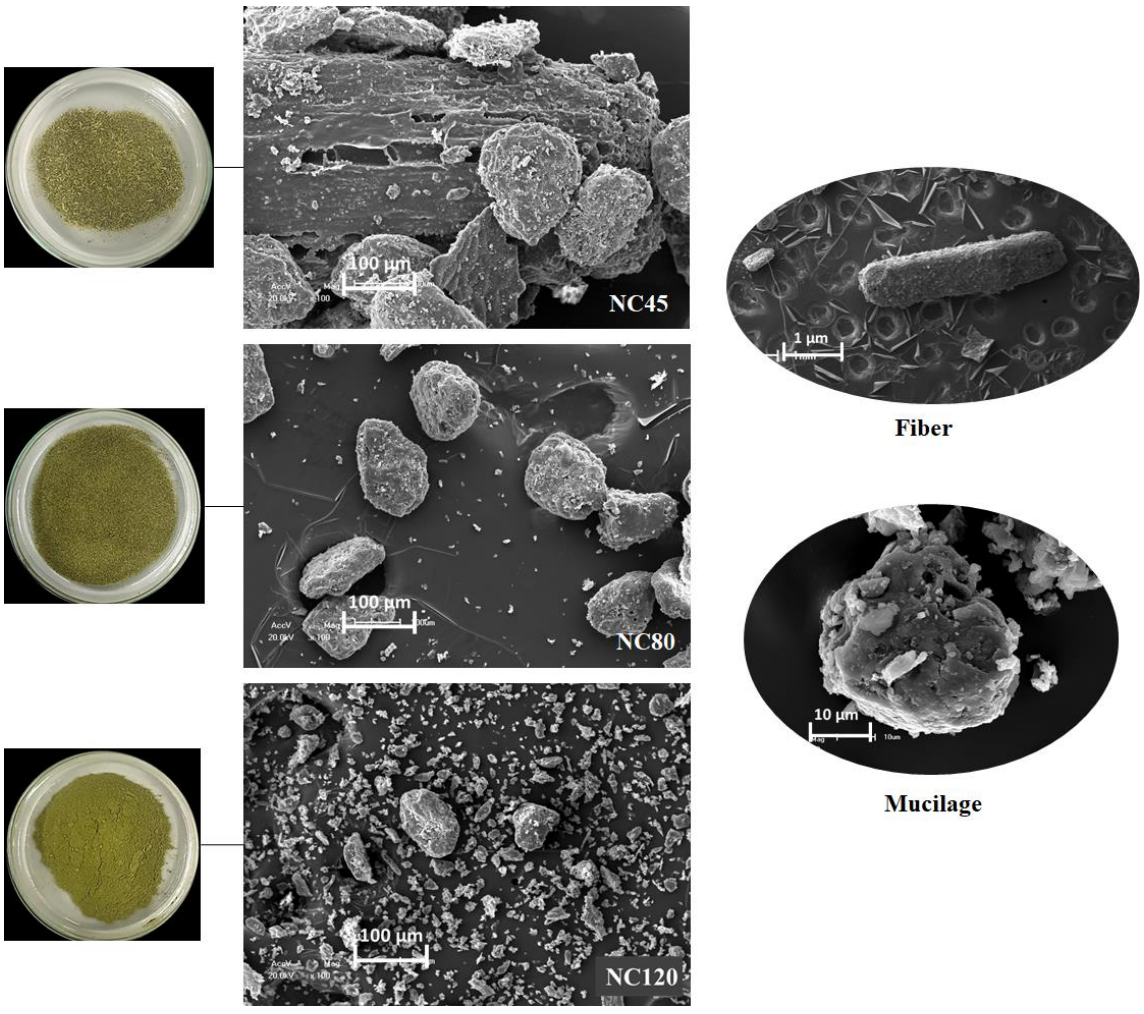

Figure 2. Scanning electron microscopy of the nopal cladodes flours NC-45, NC-80, and NC-120 at 100x magnification.

3.5. *Infrared absorption spectroscopy (FT-IR)*

As observed in Figura 3, NC-45, NC-80, and NC-120 had similar FT-IR spectra, with slightly different peak intensities, mainly in the case of the broad absorbance detected in the region of 3292 cm^{-1}. This band is commonly associated with stretching of the O-H axial bond related to alcohol and carboxylic acid involved in intermolecular OH bonding (Gheribi et al., 2018). The relative intensity of the band was significantly higher for the formulations NC-45 and NC-80, indicating that these fractions have higher water retention capacity (Guadarrama-Lezama et al., 2018). Wich is also demonstrated by the higher moisture content as shown in Table 1.

The spectra of NC-45, NC-80, and NC-120 displayed bands at 2915 and 2935 cm^{-1}, which we assigned to symmetric and asymmetric C-H stretching. These peaks are mainly related to the presence of protein, cellulose, and lignin (Maniglia & Tapia-Blácido, 2019; Lefsih et al., 2017; Abidi, Cabrales & Haigler, 2014). Therefore, the presence of these bands confirmed the presence of fibers and protein in NC-45, NC-80, and NC-120, that it is in conformity with the chemical composition (Table 1). Both absorption bands could also be attributed to functional groups of neutral polysaccharide mucilage components, such as arabinose, rhamnose, galactose, and xylose (Gheribi et al., 2018; Madera-Santana et al., 2018). These results corroborating the presence of carbohydrates in the mucilage fraction of the flours, wich is in agreement with the obtained in table 1.

The spectra of NC-45, NC-80, and NC-120 also presented an intense peak at 1614 cm^{-1}, which we attributed to vibrations of esterified carboxyl groups and free carboxyl groups. Some authors have analyzed nopal mucilage by FTIR. For example, Contreras-Padilla et al. (2016) reported a carbonic signal at approximately 1600 cm^{-1}, whilst Otálora et al. (2019) observed a peak at 1636 cm^{-1}, which they ascribed to C=O stretching, COO^- stretching, and COO^- asymmetric vibration possibly caused by the presence of uronic acid (Luo et al., 2009; Mohan et al., 2012). Therefore, the peak at 1614 cm^{-1} detected herein could be attributed to

the presence of polysaccharides such as pectin (Lefsih et al., 2017; Udeh & Erkurt, 2017). The band at 1320 cm^{-1} referred to the C-H (methyl) vibration of the rhamnose ring, which is usually present in nopal mucilage (Madera-Santana et al., 2018). In addition, the peaks between 1320 and 1025 cm^{-1} are characteristic of –COOH groups, confirming the presence of aromatic proteins, phosphoric groups, and polysaccharides (Bouaouine et al., 2018). We assigned the band at 1000 cm^{-1} to C-OH and CH_2 deformation and C-O-C bond elongation (Mohan et al., 2012). The peak at 1069 cm^{-1} corresponded to pyranose stretching vibration (Ma et al., 2017). We attributed the small band at 778 cm^{-1} to the galactose absorption band as mentioned by Madera-Santana et al. (2018) for nopal mucilage.

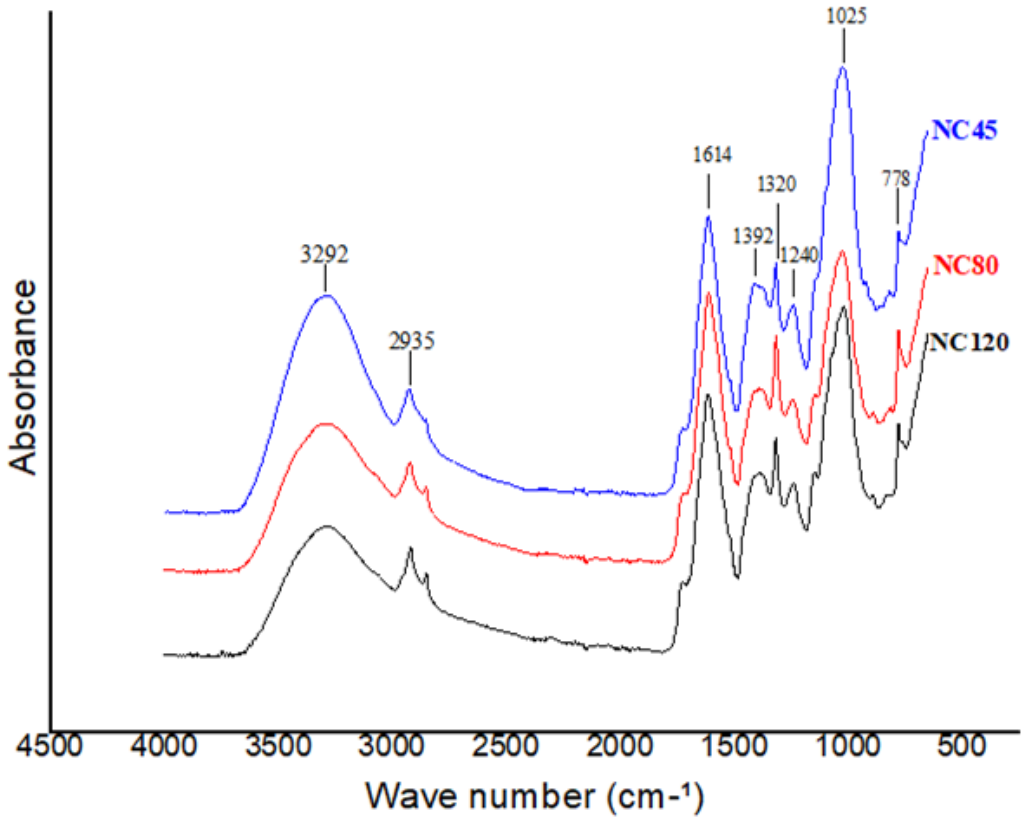

Figure 3. FT-IR spectrum patterns of the nopal cladode flours NC-45, NC-80, and NC-120.

3.5. X-ray diffraction

The X-ray diffraction patterns of NC-45, NC-80, and NC-120 displayed similar peaks at 2θ 14, 20, 24, 28, 30, 36, and 39°. Only the intensities of the peaks were different: peaks were less intense for NC-45, which was the flour with the largest average particle size (Figure 4). The diffraction patterns of NC-45, NC-80, and NC-120 resembled the patterns reported by Contreras-Padilla et al. (2015) and Rodríguez-García et al. (2007) for nopal cladode powder. The peaks in the diffractograms of NC-45, NC-80, and NC-120 could be related to the presence of calcium oxalate monohydrate (whewellite), calcium carbonate, magnesium oxide, calcium-magnesium bicarbonate, and potassium peroxydiphosphate as reported by Contreras-Padilla et al. (2015) for samples of incinerated nopal and by Madera-Santana et al. (2018) for nopal mucilage. Thus, the nopal cladode flours produced herein (NC-45, NC-80, and NC-120) can be considered a vegetable source of calcium. In addition to these crystallites, the XRD pattern of NC-45, NC-80, and NC-120 displayed peaks at 2θ = 14º and 22º, which indicated a cellulose type I crystalline structure (Yan et al., 2019), and a peak at 2θ = 24º, which belongs to hemicellulose (Li et al., 2018). These cellulose and hemicellulose peaks attested to the presence of fibers in NC-45, NC-80, and NC-120, in agreement with the micrographs in Figure 2. Contreras-Padilla et al. (2015) and Ilyas et al. (2017) emphasized that cellulose is another important crystalline structure in nopal. On the other hand, NC-45, NC-80, and NC-120 had similar relative crystallinities (~14%), showing that milling and sieving did not affect the crystalline compounds in the flours. The low relative crystallinity of NC-45, NC-80, and NC-120 could also suggest that the cellulose content of the flours was low because lignocellulosic biomass with high cellulose content also have high relative crystallinity, around 53% (Harris & DeBolt, 2008).

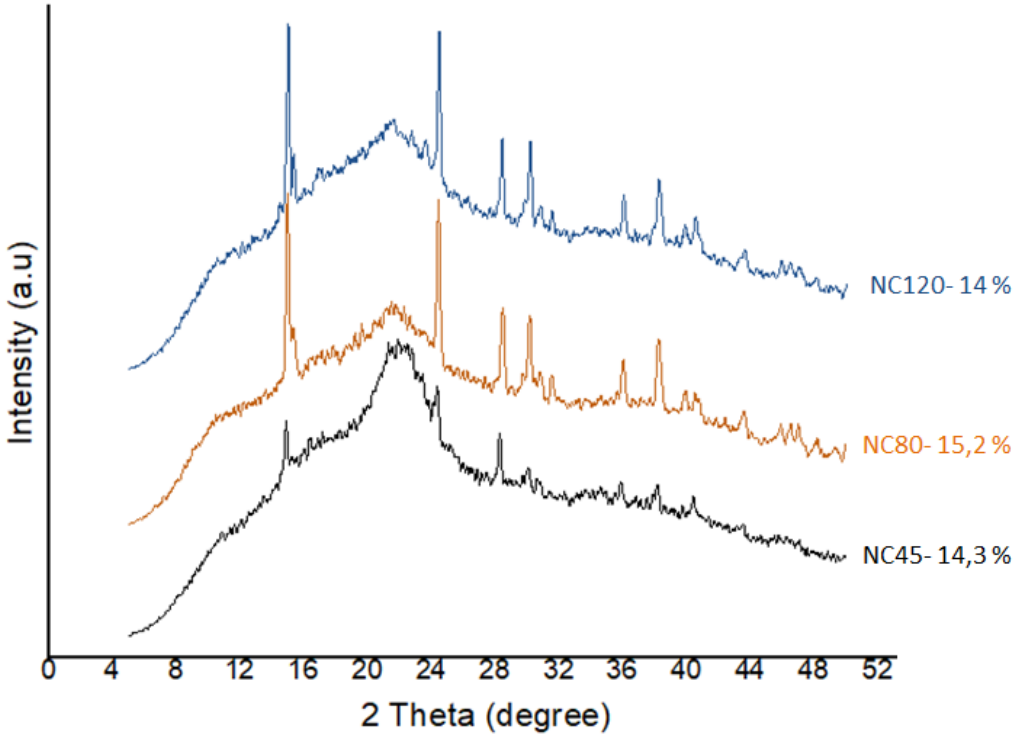

Figure 4. X-ray diffraction patterns of the flours NC-45, NC-80, and NC-120.

3.6. Application of nopal cladode flour in edible film production

NC-120 was chose as raw material for edible film production because this flour had small particle size and high antioxidant activity. The attempt to obtain a film with NC-120 alone was unsuccessful as this flour afforded a discontinuous and brittle matrix, as shown in Figure 5A. Thus, the cassava starch was added as a polymer matrix, to obtain the film the starch/NC120 film (Figure 5C). Figure 5B contains the picture of the cassava starch film (control).

The starch/NC120 film was colorful and greenish, whereas the control film was transparent and not colorful. The starch/NC flour film was more stable during storage and was more amenable to handling than the control film, which was highly hygroscopic and rapidly absorbed moisture from the environment.

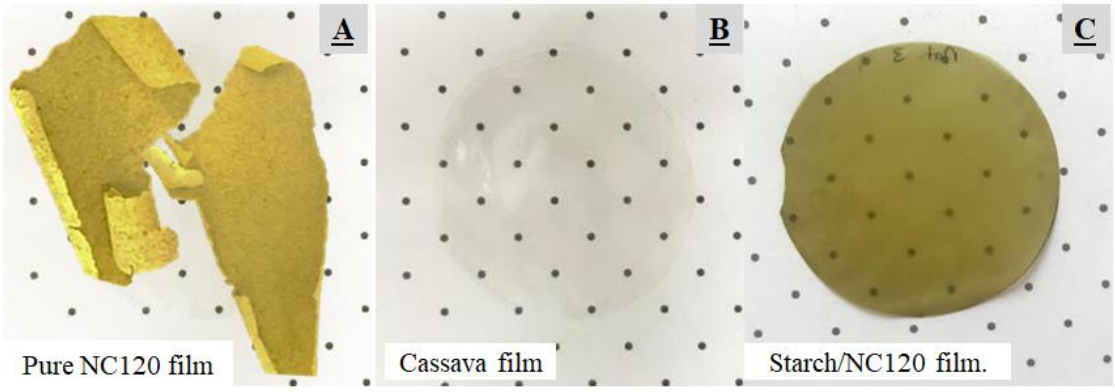

Figure 5. Visual aspect of the pure NC-120 film (A), cassava starch film (control) (B), and starch/NC120 film (C).

3.6.1. Film characterization

Table 3 depicts the mechanical properties, water vapor permeability (WVP), solubility in water, moisture, contact angle, total phenolic compound content, and activity antioxidant measurement by the DPPH and ABTS assays of the control and starch/NC-120 films. The starch/NC-120 film was thicker than the control film due to the presence of fibers in NC-120, which caused film swelling.

NC-120 addition to the cassava starch matrix yielded a weaker, less elongable, and less rigid film than the control film. Consequently, NC-120 addition to the cassava starch matrix diminished the tensile strength, elongation at break, and Young's modulus of the control film by 41.52%, 68%, and 65.52% respectively. Because NC-120 addition introduced fibers, sugars, and lipids into the cassava starch, these compounds may have disrupted the cassava starch matrix, to give a weaker film than the control film.

The control and starch/NC-120 films had similar moisture content, but different solubility and contact angle ($p < 0.05$). NC-120 addition to the cassava starch matrix produced a more soluble and hydrophilic (< contact angle) film than the control film. The sugars (mucilage fraction) in NC-120 flour might have enhanced these properties. On the other hand, NC-120 addition to the cassava starch matrix improved the water vapor barrier of the cassava starch film. Thus, the starch/NC120 film had lower WVP than the control film. This could be

justified by the presence of fibers in NC-120. This component usually increases tortuosity, thereby reducing water vapor diffusion in the film (Jensen et al., 2015, Martelli-Tosi et al., 2016). Moreover, the presence of lipids in NC-120 increased the water vapor barrier of the starch/NC120 film.

Table 3 also shows that, with NC-120 addition to the cassava starch matrix, phenolic compounds were incorporated into the film, to afford films with antioxidant activity as revealed by the DPPH and ABTS methods. The control film had very little phenolic compound content (1.62 mg of GAE/g of film), so it did not display antioxidant activity. Meanwhile, the starch/NC120 film presented high phenolic compound content (13.67 mg of GAE/g of film), and antioxidant activity as demonstrated by the DPPH (88.43%) and ABTS (7.64 µM Trolox/g of film) assays. The antioxidant activity of starch/NC120 film was higher than babassu films (~70%) (Maniglia et al., 2017).

Table 3. Thickness, mechanical and functional properties of the cassava starch and starch/NC-120 films.

Property	Control	Starch/ NC-120
Thickness (mm)	0.063 ± 0.01^{b}	0.094 ± 0.02^{a}
TS (MPa)	4.72 ± 0.65^{a}	2.76 ± 0.21^{b}
E (%)	51.76 ± 3.60^{a}	16.57 ± 0.90^{b}
MY (MPa)	183.57 ± 5.20^{a}	68.93 ± 11.9^{b}
Moisture (%)	14.83 ± 0.23^{a}	14.85 ± 0.15^{a}
Solubility (%)	20.35 ± 2.13^{b}	32.13 ± 0.10^{a}
WVP*	2.68 ± 0.04^{a}	1.09 ± 0.02^{b}
Water contact angle (Degree)	51.11 ± 0.11^{a}	19.66 ± 0.10^{b}
Total Phenolics (mg of GAE/g of film)	1.62 ± 0.1^{b}	13.67 ± 0.5^{a}
DPPH (%)	Traces	88.43 ± 4.19
ABTS (µM Trolox/g of film)	Traces	7.64 ± 0.60

Values with different letters in the same column are significantly different according to Tukey's test ($p < 0.05$).
*WVP: Water Vapor Permeability ($*10^{-10}$ $g.m^{-1}.s^{-1}Pa^{-1}$).

3.6.2. M*orphology by SEM of films*

Figure 6 displays the micrographs of the surface and cross-section of the control and starch/NC120 films. On the basis of the SEM images, the film microstructures differed widely. The cassava starch film presented smoother and more regular surface, whereas the starch/NC120 film exhibited rougher and less dense surface. The presence of fibers in NC-120 could explain the irregular surface of the starch/NC-120 film. The cross-section of the films showed the less compact and discontinuous structure of the starch/NC-120 film, which could indicate inefficient NC-120 incorporation into the cassava starch film and account for the poor mechanical properties of the starch/NC-120 film compared to the control film.

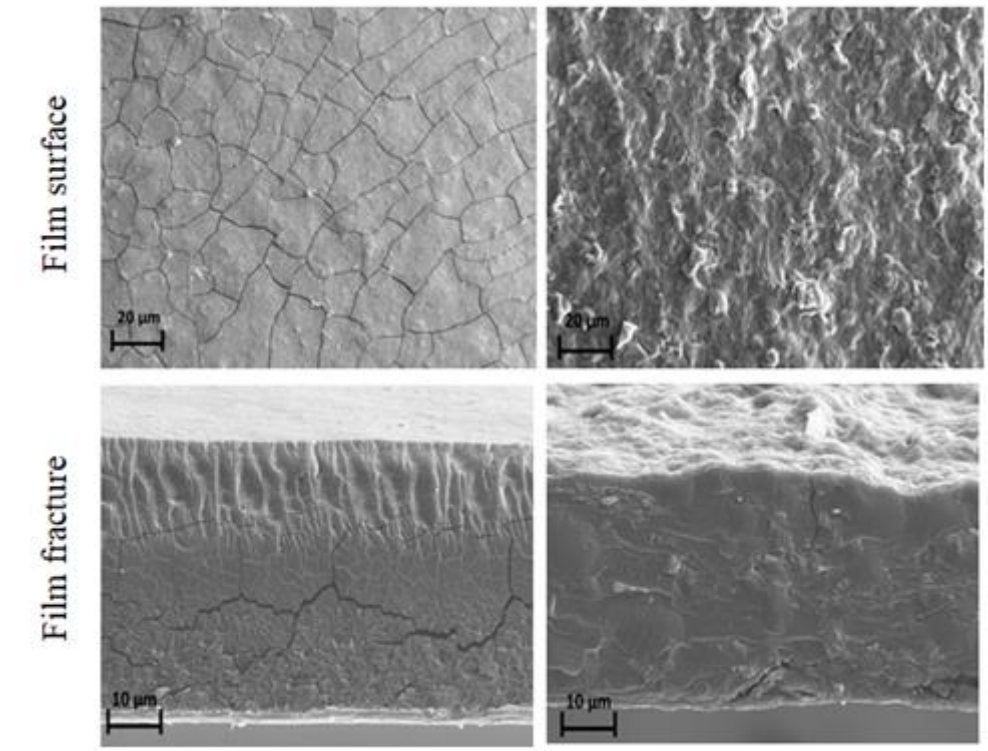

Figure 6. Scanning electron microscopy images of the surface (500 x) and cross-section (1500 x) of the cassava starch (control) and starch/NC120 films.

4. Conclusion

Milling and sieving nopal cladodes produced flours with average particle size ranging from 166 (NC-120) to 491 µm (NC-45), and with different carbohydrate and lipid contents and antioxidant capacity. NC-120 presented lower carbohydrate and higher lipid contents as well as higher antioxidant activity by the ABTS assay than NC-45 and NC-80. Moreover, microstructure by SEM revealed the presence of fibers, whereas XRD showed peaks due to cellulose type I and hemicellulose in the flours. FTIR analysis evidenced the presence of sugars in the flours. NC-120 did not form a film due to the complex composition of nopal cladode flour (fiber, lipids, sugars, and proteins). However, NC-120 interacted well with the cassava starch matrix, yielding a homogeneous and easy-to-handle starch/NC-120 film. The cassava starch film added with NC-120 was weaker, less elongable, more soluble, and more hydrophillic than the cassava starch film, but NC-120 addition provided the cassava starch matrix with phenolic compounds, which enhanced the antioxidant activity of the film.

NC-120 addition also provided the matrix with fibers and lipid, which diminished water vapor permeability. In conclusion, nopal cladode flour can be used as an additive to improve the antioxidant properties of starch-based films and produce bioactive films. Future studies will deal with additional treatment of nopal cladode flour to boost flour incorporation into the cassava starch matrix, so as to enhance the mechanical properties of the starch/NC film even further.

5. Acknowledgments

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APPENDIX B - PAPER 2

EFFECT OF ALKALINE TREATMENT OF NOPAL CLADODES TO ITS APPLICATION IN THE PRODUCTION OF EDIBLE FIM

Abstract

Nopal cladode flour (NC flour-120) subjected it to alkaline treatment at different pH values (7, 8, 9, 10, 11, and 12). The NC flours treated at different pH were characterized and then added to cassava starch-based films; glycerol was used as plasticizer. The physical-chemical and functional properties of the resulting starch/NC flour composite films were evaluated. The NC flour fibers impaired the starch-starch interactions in the cassava starch film, thereby yielding films with discontinuous regions and improve the maximum temperature for use of starches as revealed by the SEM micrographs and DSC. The starch/NC flour films were less rigid and less mechanically resistant than the initial cassava starch film. NC flour treated at pH 12 afforded more elongable starch/NC flour films with a more hydrophobic surface. The X-ray diffraction patterns of the starch/NC flour films presented crystalline peaks, which were related to the presence of minerals and cellulose in NC flour. The starch/NC flour films did not exhibit antimicrobial activity, which could be justified by the presence of sugars resulting from alkaline treatment. Nevertheless, these films contained phenolic compounds, which provided high antioxidant activity as measured by ABTS (30.04 μM Trolox/g of starch/NC flour film (pH 12)) and ability to inhibit DPPH that varied from 39.94 to 88.43%. Thus, the incorporation of NC flour as a natural antioxidant appears to be a potential strategy to be used as an additive in packaging materials.

Keywords: nopal, cassava starch, bioactive activity, antioxidant activity, biodegradable film.

1 Introduction

Interest in developing biodegradable materials that can replace synthetic plastics has grown due to the pollution issues caused by plastics (Farhan and Hani, 2017; Parra et al., 2004; Sessini et al., 2016; Zhu et al., 2016). Among natural polymers, starch has been the most investigated for the production of edible films: it is abundant in nature, biocompatible, biodegradable and non-toxic (Afolabi, et al., 2012; Jacquot et al., 2014; Gross & Kalra, 2002; Yuliana, et al., 2012). However, starch films have poor mechanical properties and absorb water easily (Dai, Zhang, & Cheng, 2020). Furthermore, few types of starch display bioactive properties, which is a desirable characteristic for food packaging. Some authors have reported that the starches isolated from babassu mesocarp, annatto, and turmeric residue exhibit antioxidant activity (Maniglia et al., 2017; Maniglia et al., 2019; Silveira & Tapia-Blácido, 2018).

Cassava is an important tuber in Brazil. This country holds a prominent position in the world cassava production—23.07 million tons of cassava in 2017—and Brazil was behind only Nigeria, which produced 41.53 million tons (FAO, 2019). Cassava starch has been widely used to prepare biodegradable films (Bher, Auras, & Schvezov, 2018; Costa, et al., 2017; Jaramillo et al., 2016; Piñeros-Hernandez et al., 2017), but this starch presents limitations such as high hygroscopicity and no bioactive property (Jiménez et al., 2012). Therefore, we propose employing a new raw material, *Opuntia ficus-indica* cladode flour, which can add bioactive properties to cassava starch films.

Opuntia ficus-indica belongs to the family *Cactaceae.* This species comprises four main parts: cladodes (stems), flower, fruit, and seeds. This cactus plant adapts to the environment easily and occurs in many geographic areas and habitats including the Mediterranean basin, the Middle East, South Africa, Australia, South America, Central

America, North America, and India (Gheribi et al., 2018; Gheribi et al., 2019; Hernández-Hernández et al., 2011).

Cladodes (succulent stem), known as pads or nopals, are composed of water (80–95%) as well as small amounts of carbohydrates (3–7%), fiber (1–2%), and protein (0.5–1%) (Zhao et al., 2007). The sugar moiety includes mucilaginous components that contain polymers, like (1-4)-linked β-D-galacturonic acid chains and R(1-2)-linked L-rhamnose residues (Lee et al., 2003; Trachtenberg & Mayer 1981). Cladodes also represent a source of phytochemicals, such as phenolic acids (aromadendrin, taxifolin or dihydroquercetin, isorhamnetin, vitexin, kaempferol, quercetin, betalains, betacyanins, rutin, and isorhamnetin) and flavonoids, and they contain citric and malic acids and some glucose and fructose content (Andreu et al., 2018; Blando et al., 2019; de Wit et al., 2019; Ginestra et al., 2009; Osuna-Martínez, Reyes-Esparza, & Rodríguez-Fragoso, 2014). For this reason, some authors highly recommend that cladodes be included in the human diet to improve the gastrointestinal health of suckling sows (Ortiz et al., 2019). Additionally, nopal cladodes contain mucilage and cellulosic fibers.

Mucilages and pectins have been extracted from the cladodes and peels of prickly pears and have been applied as thickening, gelling, emulsifying, and film-forming agents in foods, cosmetics, and pharmaceutical and personal-care products. Some authors have suggested that nopal mucilage can be used as a novel bioplastic and biomaterial for food packaging and biomedicine (Ayadi et al., 2009; Sáenz, Sepúlveda, & Matsuhiro, 2004; Scognamiglio et al., 2019;). Mucilage extracted from cladodes have also been successfully employed as coating to increase the shelf life of highly perishable fruits like kiwi fruit and ‘Dottato’ fig fruit (Allegra et al., 2016; Allegra et al., 2017). Furthermore, mucilage, which is hydrophilic, has been applied to produce films with elevated water vapor permeability even without the addition of a plasticizer. Moreover, mucilage films produced at native pH present

the best barrier to water vapor and the highest tensile strength (Espino-Díaz et al., 2010; Gheribi et al., 2018). In addition, cladode cellulosic fibers have been used as reinforcing agent of thermoplastic starch based on potato starch and glycerol (Scognamiglio et al., 2019). Nevertheless, research into edible films containing cladode flour has not yet been conducted.

Some strategies have been proposed in order to obtain less rigid fibers, which favor the preparation of more homogeneous dispersions (Chinga-Carrasco, 2011; Li, Zhao, & Liu, 2013). The chemical pre-treatment of the fibers using lower cost chemicals is an economically viable alternative that can afford suitable results (de Souza Fonseca et al., 2019). In this way, we have hypothesized that the addition of nopal cladode flour submitted to alkaline pre-treatment can improve the physical properties of cassava starch and provide it with bioactive properties. In this context, this work aims to evaluate how the addition of untreated or chemically treated nopal cladode flour affects the physical and bioactive properties of cassava starch-based films.

2. Methodology

2.1. Raw material

The *Opuntia ficus-indica* cladodes were freshly harvested in the Northeastern region of Brazil at the plantation belonging to the Animal Science Department of the Federal University of Ceará (UFC). Spineless cladodes were cut into cubes and dried in an oven with forced air circulation (60 °C/12 h). The material was ground and sieved through 120-mesh sieves to obtain the nopal cladode flour (NC flour). The obtained NC flour contained 10.9 ± 0.79 % of moisture, 21.4 ± 0.16 % of ash, 2.9 ± 0.37 % of lipids, 3.3 ± 0.10 % of protein, and 72.4 ± 0.60 % of carbohydrates.

2.2. Film preparation

Film forming solutions were prepared with the most refined NC flour (NC flour-120) and incorporated into a cassava starch matrix. First, NC flour was submitted to alkaline treatment at pH 7, 8, 9, 10, 11, or 12 by addition of a 0.1 M NaOH solution. All the dispersions were left in the dark and under agitation for 12 h, which was followed by filtration. The filtrate was added to the cassava starch suspension (4.3 % w/w). Glycerol at 25% concentration (w/w, based on the cassava starch weight) was added as plasticizer. The suspensions were heated at 70 ± 2 °C until a semi-viscous solution emerged. The prepared mixtures were cast onto acrylic plates and dried in an oven (BOD, Marconi) at 30 °C overnight. A cassava starch film containing NC flour at native pH (4.5) was also prepared and labeled NC flour (native). A cassava starch film was obtained and used as control (named control film). The dried films were conditioned in a desiccator at 25 °C and 53% RH for 48 h.

The average thickness of the preconditioned films (58% RH, 25 °C) was calculated from three measurements at random positions; a Zaas-Precision flat tip digital micrometer (1-μm resolution) was employed.

2.3. Scanning electronic microscopy (SEM)

The morphology of films was analyzed by Scanning Electron Microscopy (ZEISS model EVO-50). Each sample was covered with gold by sputtering (Bal-Tec SCD 050) and accelerated under 20 kV.

2.4. Film mechanical properties

The mechanical tensile strength of films was measured on a TA Texturometer TX Plus (TA Instrument) according to ASTM D882–D12 (2012). The films were cut into 2.54 cm-wide and 10 cm-long strips; five samples of each formulation were analyzed. The initial grip separation and the crosshead speed were set at 80 mm and 1.0 mm s^{-1}, respectively. The tensile strength (force/initial cross-sectional area) and the elongation at break were determined

directly from the stress × strain curves by using the software Texture Expert V.1.22 (SMS, Surrey, U.K.). Young's modulus was calculated as the slope of the initial linear portion of this curve.

2.5. Film functional properties

2.5.1 Moisture

The moisture content of the films was gravimetrically determined. The films were dried in an oven at 105 ^{o}C for 24 h, and their moisture content was calculated according to ASTM D644-99 (ASTM 1999).

2.5.2 Solubility

Film solubility in water was calculated as the percentage of dry matter of the solubilized films (discs with diameter of 20 mm) after immersion in 50 mL of water at 25 ± 2 °C for 24 h. The non-solubilized fractions were separated by filtration and dried at 50 °C until constant weight, and the percentages of dry matter were calculated (Gontard, Guilbert & Cuq, 1992).

2.5.3 Water Vapor Permeability

The water vapor permeability (WVP) was gravimetrically measured according to the ASTM Method E96/E96M (ASTM, 2014) at 25 ± 2 °C, as described by Martelli-Tosi et al. (2016). The films were sealed over a permeation cell containing silica gel and placed in desiccators containing distilled water until steady-state conditions were reached. The cell was weighed every 1 h for 9 h. WVP was calculated as WVP = w.x/t.A.P, where "x" was the average thickness of the films, "A" was the permeation area (0.00196 m^2), "P" was the difference between the partial pressure of the atmosphere over silica gel and over pure water (3.168 kPa, at 25◦C), and the term w/t was calculated by linear regression from the data of weight gain as a function of time.

2.5.4 Water Contact Angle

The wettability of the films was analyzed by measuring the contact angle with OCA-20 Dataphysics (Germany). The water contact angle is defined as the angle between the water drop and the film surface (Ojagh et al., 2010) and is expressed in degrees. All the analyses were accomplished in triplicate.

2.6. FT-IR analysis

Fourier Transform Infrared (FTIR) analysis of films was accomplished to determine their functional groups. The spectra were recorded from 500 to 4000 cm^{-1} on an FTIR Spectrometer Shimadzu, IR Prestige-21 (Kyoto, Japan) with Fourier transform at a nominal resolution of 2 cm^{-1}.

2.7. X-ray diffraction analysis

X-ray diffraction analysis of films was performed on a Siemens X-ray diffractometer (Siemens, model D5005, Baden-Württemberg, Germany) operating at 40 kV and 30 mA with Kα copper radiation and monochrome filter at room temperature. The data were collected from 2 to 50 °C on a 2θ scale with speed of 0.02 °/ s. The crystallinity index (%) of the materials was quantitatively estimated as the ratio of the crystalline area of the individual peaks by the total area of the total diffractogram area; the Nara & Komiya (1983) method was followed, and Origin 8.1 software was used (OriginPro Corporation, Massachusetts, USA).

2.8. Differential scanning calorimetry (DSC)

The thermal behavior of the films was studied by differential scanning calorimetry DSC TA 2010 equipment controlled by a TA5000 module (TA Instruments, New Castle, DE, EUA) attached to a cryoscopic cooling accessory. Approximately 5 mg of the dry film sample was placed in DSC pans that were sealed. All measurements were performed at a heating rate of 10 °C min−1 from -150 °C to 150 °C under a nitrogen atmosphere (45 mL min−1).

Thermograms were evaluated using Universal Analysis 2000 software (TA Instruments, New Castle, USA).

2.9. Total phenolics content analysis

The total phenolics content of films was determined according to Larrauri, Rupérez, & Saura-Calixto, (1997) with some modifications. To this end, 0.5 g of NC flour treated at a certain pH or 0.5 g of a Starch/NC film was mixed with 9.5 g of distilled water (5% w/w) under slight stirring and heated at 40 °C for 10 min. The resulting extract was filtered and stored. Next, 30 µL of extract was mixed with 470 µL of distilled water, 500 µL of Folin-Ciocalteau solution (1:3), 1 mL of sodium carbonate (20%), and 1 mL of distilled water, in this sequence. Then, the mixture was incubated in the dark for 30 min. The absorbance was measured at 700 nm, and the result is expressed as mg of gallic acid equivalent per g of sample (mg GAE/g of NC flour and mg GAE/g of starch/NC flour film).

2.10. DPPH radical scavenging activity analysis

To evaluate the anti-radical activity of films the DPPH method was conducted according to Martins, Cerqueira, & Vicente (2012). The sample extract was obtained by using 200 mg of each starch/NC flour film mixed with 2 mL of deionized water followed by stirring at 25 °C for 3 h. Then, 500 µL of the supernatant was mixed and reacted with 2 mL of DPPH radical (0.06 mmol/L) and incubated at 25 °C in the dark for 30 min. The control consisted of 500 µL of methanol without film. After that, the absorbance was read at 517 nm with a spectrophotometer (HP Hewlett Packard 8453, USA). The results were computed as follows (see Eq. (1)):

$$AC\% = \left(1 - \frac{A\ sample}{A\ control}\right) \times 100 \qquad \textbf{(1)}$$

where AC is the antioxidant capacity, and A_{sample} and $A_{control}$ are the absorbance of the sample and control, respectively. All the experiments are carried out in triplicate. The antioxidant capacity of films is expressed in %DPPH radical scavenging activity/100 mg of film.

2.11. ABTS radical scavenging activity analysis

The antioxidant activity was measured by analyzing the $ABTS^{\cdot+}$ radical scavenging activity according to Re et al. (1999) and Rufino et al., (2007) with some modifications. The radical cation $ABTS^{\cdot+}$ was produced by reacting 5 mMol L^{-1} $ABTS^{\cdot+}$ stock solution with 144 mMol L^{-1} potassium persulfate in the dark at room temperature for 16 h before use. The $ABTS^{\cdot+}$ solution was diluted with ethanol until an absorbance of ~ 0.70 at 734 nm was achieved. Then, 30 μL of sample was added to 3 mL of the diluted $ABTS^{\cdot+}$ solution, and the absorbance was read at 734 nm with a spectrophotometer (HP Hewlett Packard 8453, USA) after incubation at room temperature for 10 min. The antioxidant capacity is expressed as μM Trolox equivalent antioxidant capacity per g on dry basis (μM Trolox g^{-1} db).

The sample extract was prepared by using 200 mg of the sample and 4 mL of deionized water. The mixture was left in the dark at room temperature for 3 h before analysis.

2.12. Microbiological assay

Films antimicrobial activity were evaluated by the agar diffusion assay according to Pelissari et al. (2009) and Shapi'i et al. (2020), with some modifications; Four microorganisms were analyzed: three bacteria (*Staphylococcus aureus, Escherichia coli,* and *Pseudomonas aeruginosa*) and one yeast (*Candida albicans).* For this test, the cassava starch film was used as control. The nopal extract was also tested (20 uL/disc). The films were cut into discs with diameter of approximately 1.5 cm. Subsequently, the films were exposed to UV light for eight minutes each side. Each film or disc was aseptically placed on the surface of the previously inoculated Tryptone soya agar (TSA) culture medium with 100 μL of

suspension (MacFarland scale 1) of each microorganism. The plates were incubated at 37 °C for 24 h. For all the trials, the cassava starch film was tested as negative control.

2.13. Univariate statistical analysis

The data concerning to starch/NC flour films were subjected to one-way ANOVA analysis with 95% significance level by using the Statistica software (version 7.0). Tukey's test was performed to determine significant differences (< 0.05) between between film properties.

3. Results and discussion

3.1. Films visual aspect

Figure 1 displays pictures of the cassava starch film (designated control film hereafter) and of the starch/NC flour composite films. The control film was transparent, but NC flour addition afforded colored films: the starch/NC flour films were greenish. In addition, the starch/NC flour films were smooth and translucent, but it was more difficult to remove them than removing the control film because the former contained fibers. Nevertheless, the control film was highly hygroscopic and rapidly absorbed moisture from the environment, while the starch/NC flour films were more stable, which facilitated their handling.

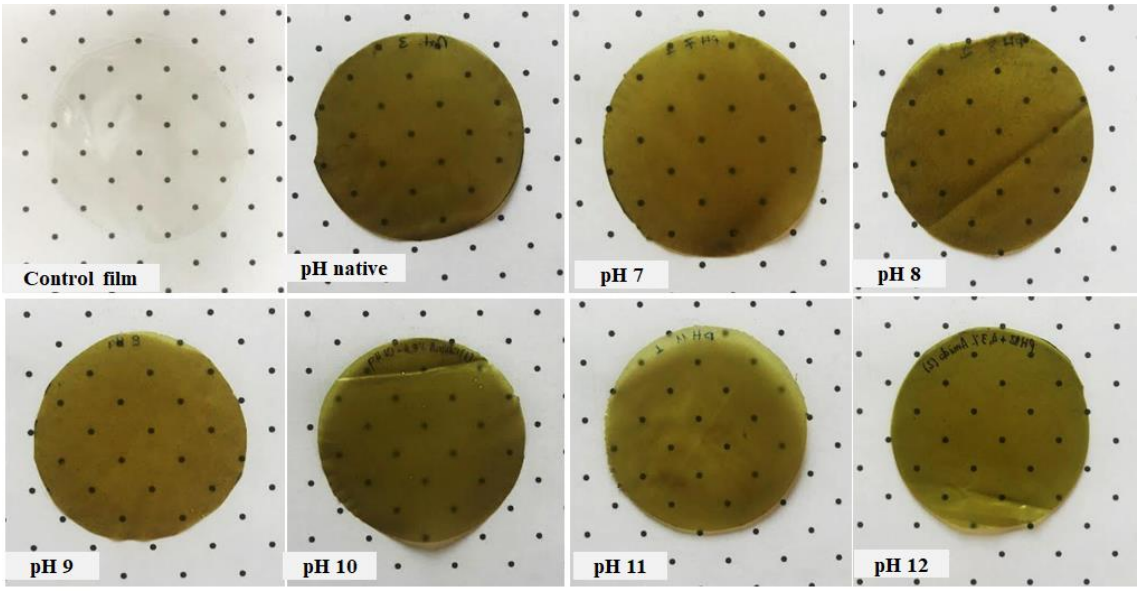

Figure 1. Visual aspect of the control film and the films based on cassava starch added with NC native and treated at pH 7, 8, 9, 10, 11, and 12.

3.2. Scanning Electron Microscopy (SEM)

Despite the color variance among the films, only slight differences in the visual aspect of the films were observed (Fig 1), however the SEM images (Fig. 2) revealed huge differences in the film structures. The control film presented a regular and continuous aspect, whereas the starch/NC flour films exhibited a discontinued structure and irregular surface due to the presence of fibers. NC flour inclusion into the cassava starch matrix caused irregularities on the film surface and provided a less compact transversal section as highlighted by the non-homogeneous thickness. On the other hand, the SEM micrograph showed that alkaline treatment of NC flour affected NC flour incorporation into the cassava starch film. Thus, different interactions between the biopolymers present in NC flour and cassava starch could arise at different pH values. The SEM micrographs of the starch/NC flour films demonstrated that pHs closer to the native pH (5) yielded films with more porous structure, whilst high pH values afforded films with fewer cracks in the transversal section and greater thickness, which could indicate swelling of the fibers at pH 10, 11, and 12 as

compared to native pH. The starch/NC flour film produced with NC flour-120 treated at pH 12, labeled starch/NC flour (pH 12) had more regular structure with fewer visible fractures.

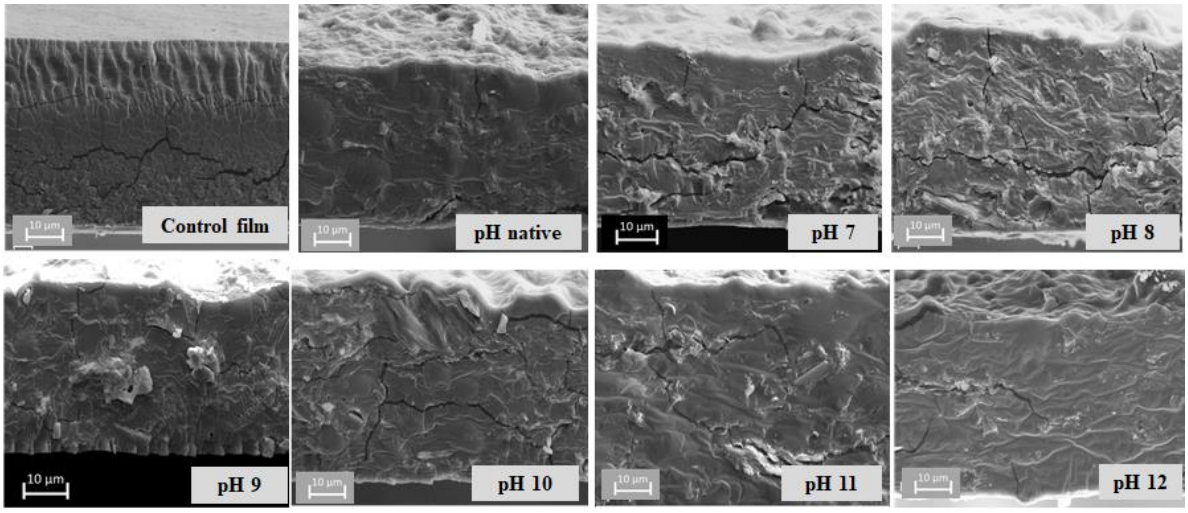

Figure 2. Scanning electron microscopy of the fractures of the control film and the films based on cassava starch added with NC native and treated at pH 7, 8, 9, 10, 11, and 12 (1500x).

3.3. Mechanical properties of films

Figure 3 shows the mechanical properties of the control film and the films based on cassava starch added with NC native and treated at pH 7, 8, 9, 10, 11, and 12, which helped to evaluate how NC addition and alkaline treatment affected film properties. NC flour addition significantly increased film thickness as compared to the control film. The presence of fibers in NC flour swelled the film and increased its solid content, thereby augmenting its thickness. The NC flour-120 alkaline treatment did not influence the starch/NC flour film thickness.

NC flour-120 addition decreased the film tensile strength (TS) and Young's modulus (Scognamiglio et al., 2020). However, elongation at break increased almost proportionally to the rise in NC flour-120 alkaline treatment pH and ranged from 16.6 ± 0.9% (starch/NC flour (native)) to 72.3 ± 12.3% (starch/NC flour (pH 12)). This agreed with the low relative crystallinity values of the starch/NC flour films revealed by X-ray diffraction. Because NC flour addition introduced fibers as well as sugars and lipids that disrupted the

cassava starch matrix continuity (Figure 2) and the starch-starch interactions, as shown in the FTIR analyses (Figure 4), the starch/NC flour film mechanical resistance was lower as compared to the control film. The compounds arising during NC flour-120 alkaline treatment could act as plasticizer in the starch/NC flour films, mainly when the treatment was performed at pH 12, which extracted pectin, protein, and lipids from nopal cladodes more effectively. As seen in the increase in the elongation at break of the cassava starch film added with NC pH 12 (Figure 3).

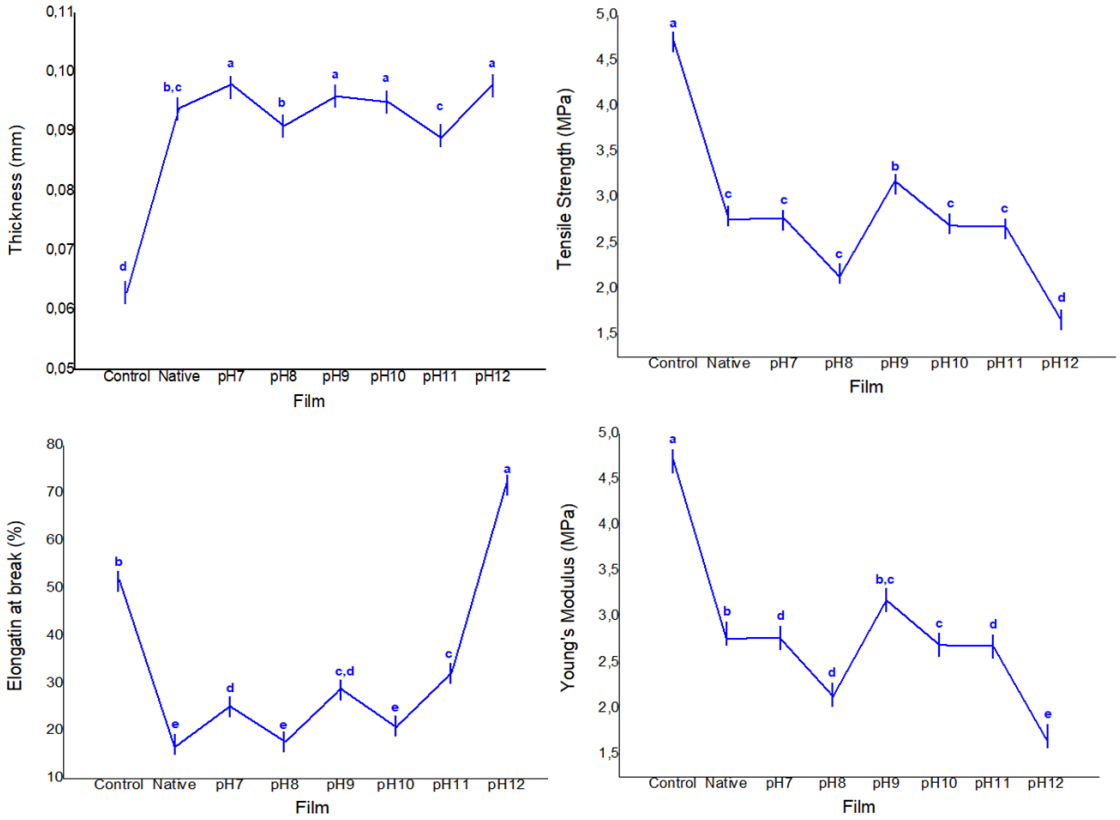

Figure 3. Thickness (mm), Tensile strength - TS (MPa), Elongation at break (%) and Young's modulus- MY (MPa) of control film (a) and of the films based on cassava starch added with NC native (b) and treated at pH 7 (c), pH 8 (d), pH 9 (e), pH 10 (f), pH 11 (g), and pH 12 (h).
Vertical bars with different letters indicate a statistically significant difference according to Tukey's test ($p < 0.05$).

3.4. Film functional properties

Table 1 presents the moisture, water solubility, water vapor permeability (WVP), and wettability of the control film and the films based on cassava starch added with NC native and treated at pH 7, 8, 9, 10, 11, and 12. NC flour-120 addition and alkaline treatment did not influence film moisture as compared to the control: all the starch/NC flour films had the same moisture (~14.8%). Meanwhile, water solubility and wettability (water contact angle) were the most affected by NC flour-120 addition and alkaline treatment.

Alkaline treatment of fibrous materials removes a certain amount of lignin, hemicellulosic compounds, pectin, pigments, lipids, and solubilized proteins and can modify the external surface of the fiber cell wall, thereby depolymerizing cellulose and exposing the short crystallites (Li, Tabil, & Panigrahi, 2007). Here, we used the filtrate of the NC flour-120 dispersion submitted to alkaline treatment to produce the starch/NC flour films. Thus, the previously mentioned compounds may have been incorporated into the starch/NC flour film matrix, impacting the functional properties of the films. In this sense, NC flour-120 addition to the cassava starch film increased film solubility in the case of NC flour-120 (pH 8–11). However, when NC flour (pH 12) was added to the film, the starch/NC flour film solubility was similar to the solubility of the control film. Therefore, more lipid compounds were extracted at this pH, or NC flour-120 (pH 12) was better incorporated into the matrix, promoting interactions between the NC flour-120 fibers, sugars, and pectin with the cassava starch.

Regarding water vapor permeability (WVP), NC flour-120 addition statisticaly decreased the starch/NC flour film WVP compared to the control film. The fibers in NC flour-120 may have made water molecule diffusion through the starch/NC flour film difficult. The presence of fibers in the matrix usually increases tortuosity, consequently reducing film

permeability (Jensen et al., 2015, Martelli et al., 2016). Moreover, the presence of lipids in the Starch/NC flour-120 films increased the water vapor barrier.

Analysis of the film contact angle evidenced that addition of untreated NC (NC flour-120 (native)) yielded a film with more hydrophilic surface (19.6°) compared to the control film (51.1°). Nevertheless, when NC flour-120 was treated at pH 7–12, the contact angle increased from 38.6 to 71.8°, indicating the more hydrophobic characteristic of the starch/NC flour film surface. The FTIR spectra confirmed the presence of other compounds bearing carboxylic groups in the starch/NC flour films compared to the control film, especially for starch/NC flour (pH 12). These groups were assigned to lipids, phenolic compounds, pectin, and sugars, which may have been more homogeneously incorporated into the starch/NC flour films as observed in the SEM micrographs (Figure 2), decreasing its wettability, and consequently its water contact angle. The starch/NC flour (pH 12) film was also less soluble.

Table 1. Functional properties of the control film and the films based on cassava starch added with NC native and treated at pH 7, 8, 9, 10, 11, and 12.

Formulation	Moisture (%)	Solubility (%)	WVP*	Water contact angle (Degree)
Control	14.83 ± 0.23^{a}	20.35 ± 2.13^{d}	2.68 ± 0.04^{a}	51.11 ± 0.11^{c}
Starch/NC-(native)	14.85 ± 0.15^{a}	32.13 ± 0.10^{c}	1.09 ± 0.02^{c}	19.66 ± 0.10^{h}
Starch/NC-pH7	14.35 ± 0.81^{a}	45.83 ± 3.69^{b}	1.10 ± 0.01^{c}	38.56 ± 0.06^{f}
Starch/NC-pH 8	14.88 ± 0.24^{a}	67.02 ± 2.68^{a}	1.98 ± 0.08^{b}	34.28 ± 0.01^{g}
Starch/NC-pH 9	14.60 ± 0.58^{a}	32.60 ± 2.78^{c}	2.59 ± 0.01^{a}	45.50 ± 0.07^{e}
Starch/NC-pH 10	14.84 ± 0.51^{a}	44.45 ± 0.97^{b}	2.37 ± 0.05^{a}	48.64 ± 0.26^{d}
Starch/NC-pH 11	14.34 ± 0.24^{a}	47.12 ± 5.21^{b}	1.22 ± 0.02^{c}	64.59 ± 0.11^{b}
Starch/NC-pH 12	15.39 ± 0.64^{a}	22.37 ± 1.00^{d}	$2.23 \pm 0.04^{a,b}$	71.79 ± 0.04^{a}

*WVP: Water Vapor Permeability ($*10^{-10}$ $g.m^{-1}.s^{-1}Pa^{-1}$).
Values with different letters in the same column are significantly different ($p < 0.05$).

3.5. Infrared absorption spectroscopy (FT-IR)

Figure 4 depicts the FT-IR spectrum of the control film and the films based on cassava starch added with NC native and treated at pH 7, 8, 9, 10, 11, and 12. Bands emerged

in three regions: from 3500 to 3200 cm^{-1} as a result of -OH stretching, from 3000 to 2800 cm^{-1} due to -CH (methyl group) stretching, and from 1300 to 1050 cm^{-1} as a common fingerprint region of polysaccharides or carbohydrate units as described by Madera-Santana et al. (2018) for polymeric materials. The main difference between the spectra of the control film and the starch/NC flour films was the shift in the band at 1643 cm^{-1} to 1614 cm^{-1} upon addition of NC flour-120 at native pH. We assigned this absorption band to carboxylate groups -C(=O)-O (Chaouch et al., 2018). It could indicate that other compounds like pectin, protein, or lipid, which bear carboxylic groups, were present in the chemically treated NC flour-120, and that other interactions were established between starch and the treated NC flour-120 (Lefsih et al., 2017; Udeh & Erkurt, 2017). The slight differences in the peak intensities of the starch/NC flour films could reflect different contents of free carboxylic groups, which would be directly related to the alkaline treatment conditions (Cárdenas, Goycoolea, & Rinaudo, 2008; Jaramillo et al., 2016;). We also observed a small band at 1483 cm^{-1} for the starch/NC flour (pH 10) film, which could be assigned to phenolic compounds.

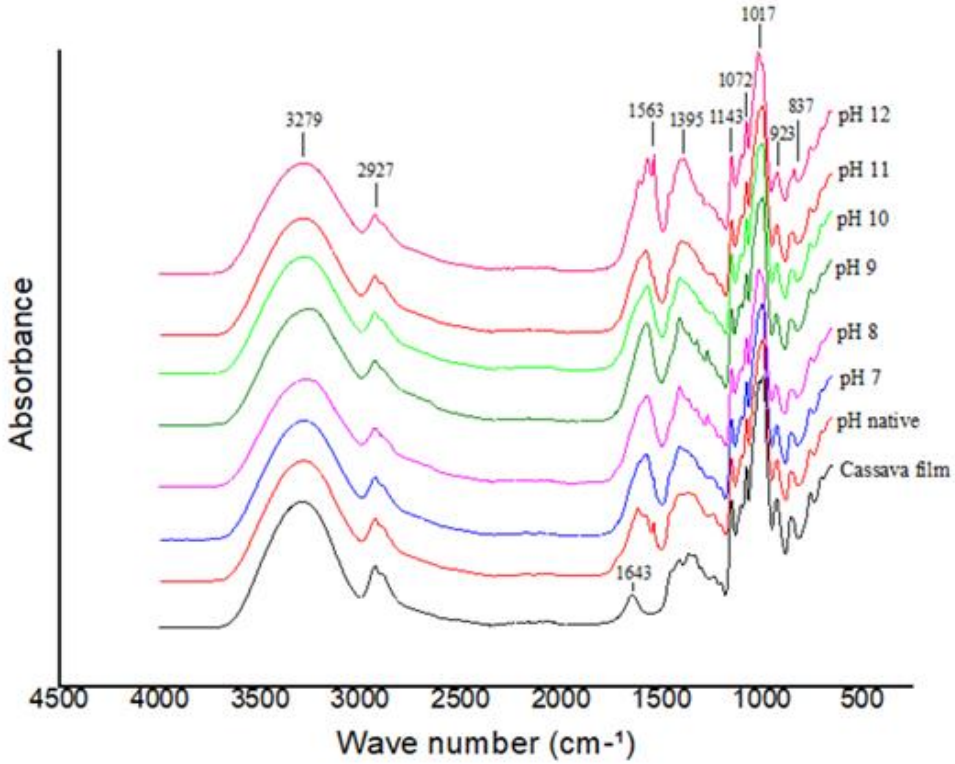

Figure 4. FT-IR spectrum patterns of control film and of the films based on cassava starch added with NC native and treated at pH 7, 8, 9, 10, 11, and 12.

3.6 X-ray diffraction of films

Figure 5 displays the diffraction patterns of the control film and the films based on cassava starch added with NC native and treated at pH 7, 8, 9, 10, 11, and 12. NC flour-120 addition to the cassava starch matrix gave rise to crystalline structures with peaks at 2θ 15, 24, and 30°, which could be related to the presence of cellulose type I. In cellulose I, the crystalline structure consists of two coexisting crystal phases: cellulose I (triclinic unit cell) and cellulose I (monoclinic unitcell), which are arranged in parallel chains (Martelli-Tosi et al., 2016; Nishiyama, Langan, & Chanzy, 2002). Regarding the relative crystallinity of the films, the values ranged from 13.4% to 17.2% for the starch/NC flour (pH 12) and starch/NC flour (pH 7), respectively. Given that the control film presented relative crystallinity of 16.8%, NC flour-120 addition altered film crystallinity. The starch/NC flour (pH 11) and starch/NC flour

films (pH 12) showed the lowest values (13.9 and 15.4 %, respectively), which suggested the presence of more amorphous zones in these formulations.

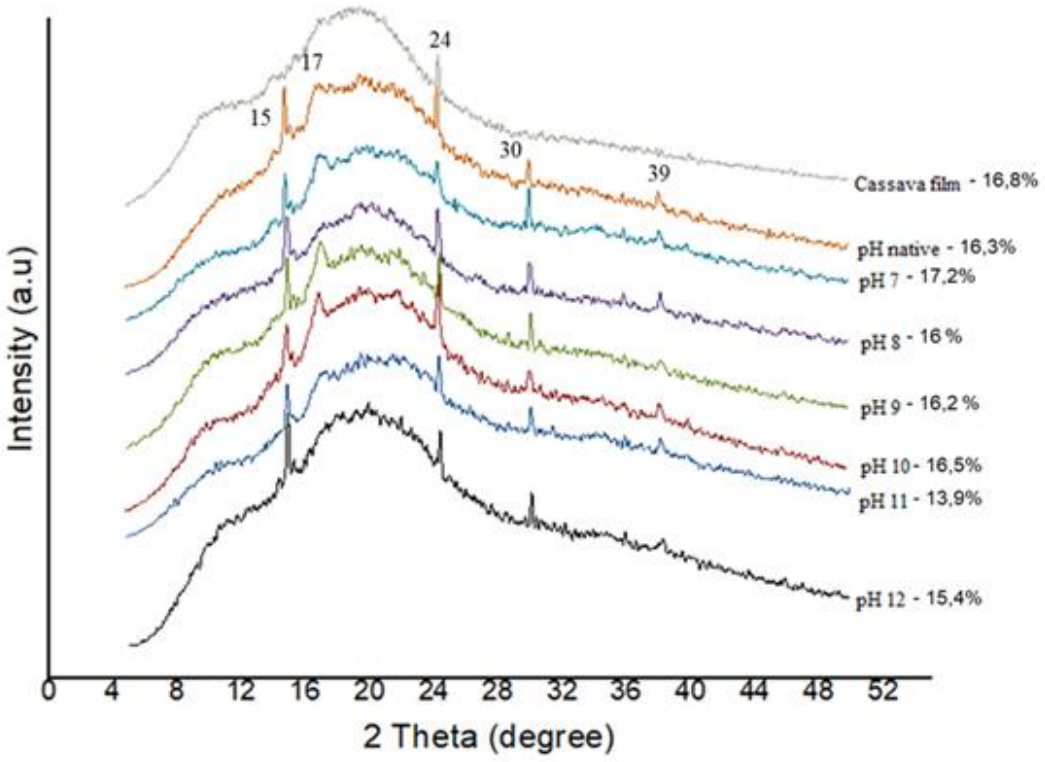

Figure 5. X-ray diffraction patterns of control film and of the films based on cassava starch added with NC native and treated at pH 7, 8, 9, 10, 11, and 12.

3.7. Differential scanning calorimetry (DSC)

Thermal stability of the cassava starch-based films was determined using DSCs. The physical properties of polymers are affected by temperature changes, thus parameters as the glass transition temperature (Tg) and the melting point (Tm) are important to determine at which their physical properties will lose their structure due to the melting and/or reassociation of the polymer structure (Abdorreza, Cheng, & Karim, 2011; Ma et al., 2019). Low and high-density polyethylene (synthetic polymers used for packaging materials), present melting temperatures of 123.6 °C and 131.7 °C, respectively (Lim et al., 2020). However biopolymers, such as cassava starch-based films must undergo sealing at similar or lower temperatures. The presence of fibers, proteins, and lipids interfere in the gelatinization process making it

difficult for water to enter inside the starch granule (Agama-Acevedo et al., 2014; Pelissari et al., 2012). Therefore, as shown in Figure 6, the thermogram showed a slight endothermic peak in approximately -70 °C (Tg) and 30 °C (Tm) for starch film (control) that shifted to -50 °C (Tg) and 85 °C (Tm) (indicated with arrows) followed by an exothermic peak at high temperature (> 100 °C). The information obtained by this preliminary investigation is useful regardless to suggest whether the introduction of nopal fibers can improve the maximum temperature for use of starches, which is presently confined at temperatures above around 30 °C, for cassava starch film. Behavior also reported by Scognamiglio et al., (2019) thermoplastic starch films added with nopal fibers. Also it is noticed that the alkaline treatment of nopal flour did not affected the thermal stability of the films.

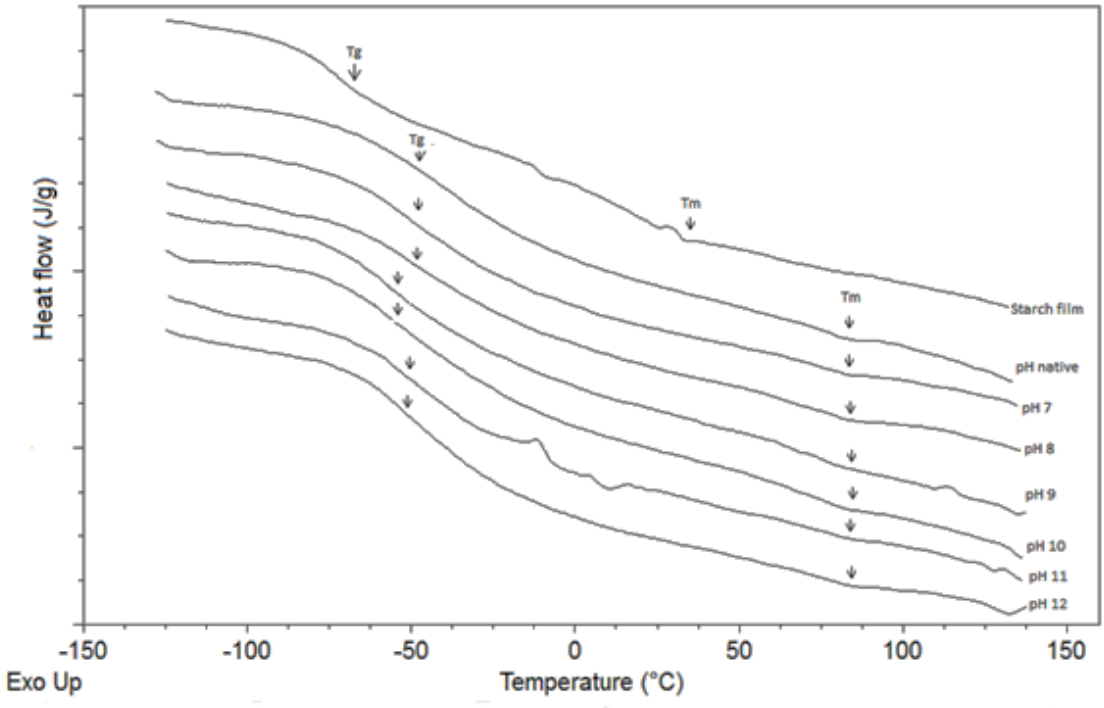

Figure 6. Calorimetry curve (DSC) of control film and of the films based on cassava starch added with NC native and treated at pH 7, 8, 9, 10, 11, and 12.

3.8. Total Phenolic compounds content and antioxidant activity of films

Table 2 depicts the total phenolics content and the DPPH and ABTS radical scavenging activity of control film and the films based on cassava starch added with NC

native and treated at pH 7, 8, 9, 10, 11, and 12. The total phenolics content in the control film was 1.62 mg of GAE/g of film, but the DPPH and ABTS assays did not reveal any antioxidant activity. The starch/NC flour films had total phenolics content ranging from 11.86 to 14.67 mg of GAE/g of film, and their ability to inhibit DPPH varied from 39.94 to 88.43%. Despite the low concentration of the NC flour-120 in the starch/NC flour films, their antioxidant capacity as demonstrated by the DPPH assay was greater than the antioxidant capacity of babassu films (~70%) reported by Maniglia et al. (2017).

The total phenolics contents in the starch/NC flour films listed in Table 5 show that the content of phenolic compounds remained the same during alkaline treatment and film preparation. Meanwhile, the starch/NC flour film (pH 12) DPPH inhibition capacity decreased. Nevertheless, analysis of the antioxidant activity by the ABTS assay pointed to an opposite behavior: starch/NC flour film (pH 12) had the highest radical scavenging activity (30.04 ± 0.56 µM Trolox/g of film). The antioxidant activity analyzed by ABTS agreed with the total phenolic content of starch/NC flour (pH 12). Therefore, alkaline treatment at pH 12 may have removed more phenolic compounds with antioxidant activity, but some of these compounds that remained in the material might have been better incorporated into the starch matrix in starch/NC flour (pH 12) and were difficult to extract by the DPPH method.

Table 2. Total phenolics determination and radical scavenging activity of control film and of the films based on cassava starch added with NC native and treated at pH 7, 8, 9, 10, 11, and 12.

Formulation	Total Phenolics*	DPPH (%)	ABTS*
Control	1.62 ± 0.1^{c}	Traces	Traces
Starch/NC-(native)	$13.67 \pm 0.5^{a,b}$	88.43 ± 4.19^{a}	7.64 ± 0.60^{d}
Starch/NC-pH7	14.19 ± 0.5^{a}	$82.55 \pm 2.42^{a,c}$	11.26 ± 0.08^{c}
Starch/NC-pH 8	$13.17 \pm 0.5^{a,b}$	$82.49 \pm 0.36^{a,b}$	18.05 ± 0.46^{b}
Starch/NC-pH 9	$12.57 \pm 0.2^{a,b}$	$80.86 \pm 0.10^{b,c,d}$	12.39 ± 1.60^{c}
Starch/NC-pH 10	$12.56 \pm 0.4^{a,b}$	$85.17 \pm 1.97^{a,b}$	12.95 ± 0.62^{c}
Starch/NC-pH 11	12.28 ± 1.4^{b}	74.64 ± 4.58^{d}	16.58 ± 1.21^{b}
Starch/NC-pH 12	11.86 ± 0.4^{b}	39.94 ± 2.79^{e}	30.04 ± 0.56^{a}

*Total phenolics are expressed as mg of GAE/g of film.

*ABTS is expressed as µM Trolox/g of film.

Values with different letters in the same column are significantly different ($p < 0.05$).

3.9. Microbiological assay of films

Figure 7 illustrates the agar diffusion assay for microbiological analysis. We assessed the antimicrobial activity of the NC extract, the cassava starch film (control) and the films based on cassava starch added with NC native and treated at pH 7, 8, 9, 10, 11, and 12 against four microorganisms: *Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa*, and *Candida albicans*. None of the films or the NC extract displayed antimicrobial activity. Furthermore, we observed larger microbial growth on the surface of the starch/NC flour films compared to the control film. This result agreed with the report by Ginestra et al., (2009) who studied the antimicrobial action of phytochemical fractions of nopal cladodes by minimum inhibitory concentration (MIC) tests, to find no antimicrobial activity against the bacteria *Escherichia coli*, *Salmonella enterica*, *Pseudomonas putida*, *Listeria innocua*, *Lactococcus lactis*, and *Staphylococcus aureus* or against the yeast *Saccharomyces cerevisiae*. The carbohydrates of nopal cladodes are present in the form of cellulose, hemicellulose, and mucilage (Kuloyo et al., 2014). However, as shown in the FTIR spectra (Fig. 4), alkaline treatment may have removed other compounds with carboxylic groups such as monomeric units (galactose, glucose, and fructose), leaving more protein

available for microorganism growth. López-Domínguez, Ramírez-Sucre, & Rodríguez-Buenfil., (2019) also found satisfactory growth of two microorganisms (*Kluyveromyces marxianus* and *Saccharomyces cerevisiae*) analyzed in culture medium enriched with nopal cladodes flour.

Therefore, we assumed that carbohydrates were present at higher concentration than phenolic compounds in the starch/NC flour films, which promoted bacterial and yeast growth.

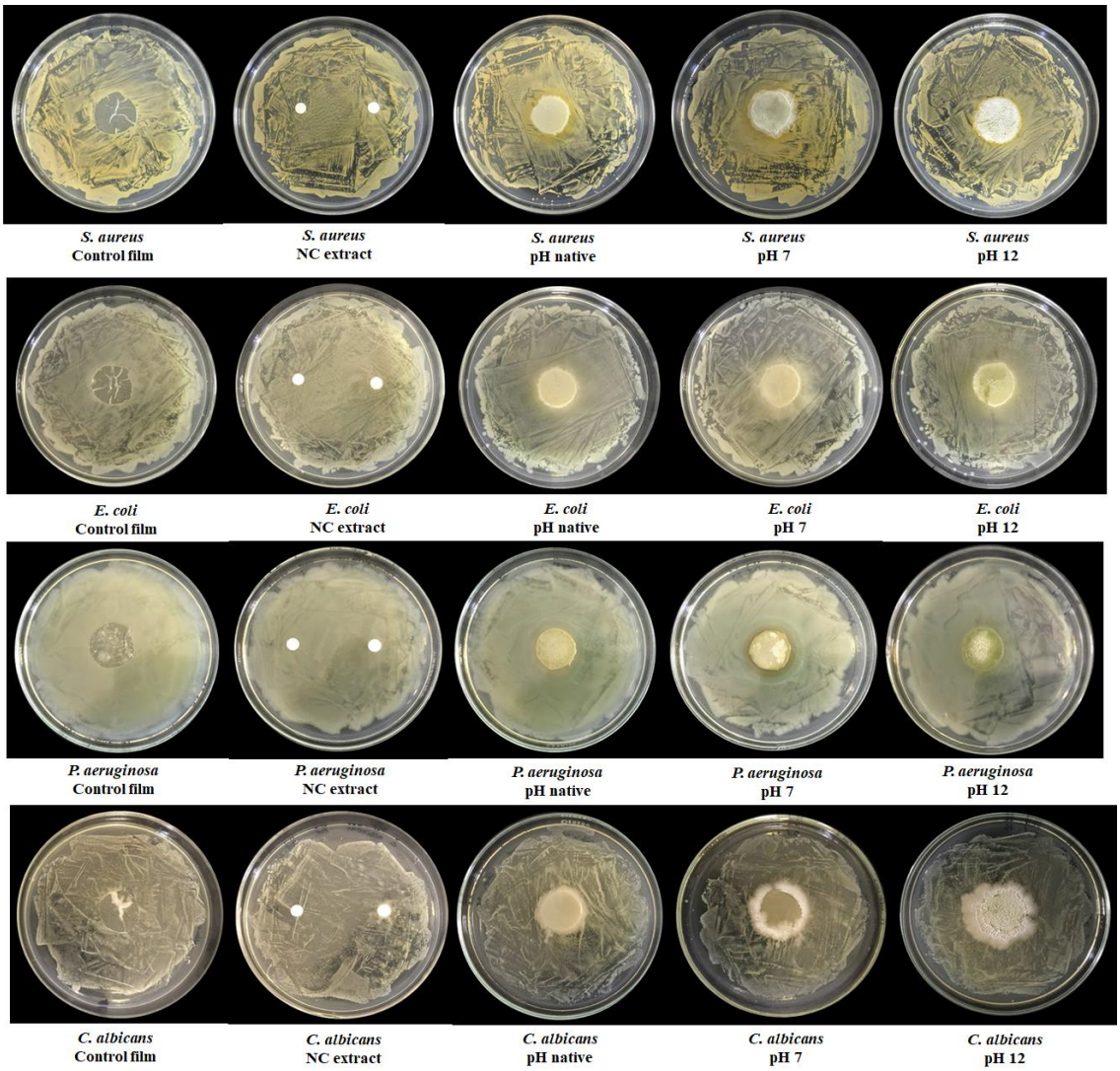

Figure 7. Microbiological assay results for the NC extract obtained after alkaline treatment (20 uL/disc), of control film and of the films based on cassava starch added with NC native and treated at pH 7, 8, 9, 10, 11, and 12.

4 Conclusion

Nopal cladode flour (NC flour-120) alkaline treatment at high pH (12) modified the surface of fibers more effectively, yielding more elongable, less soluble, more homogenous, more hydrophobic, and less wettable films as compared to the cassava starch film. NC flour alkaline treatment did not significantly affect the phenolic compounds content, so the starch/NC flour films displayed antioxidant activity. However, the high content of compounds such as sugars and protein in the NC flour-120 allowed bacteria and yeasts to grow in the starch/NC flour films surfaces.

Therefore, the starch/NC flour films did not exhibit antimicrobial activity, but is potentially useful for the production of active packaging materials, since presented high antioxidant activity as measured by ABTS and DPPH radical scavenging. The addition of nopal flour also has the ability to enhance the thermal stability of the polymer blends.

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APPENDIX C - PAPER 3

(PUBLISHED IN THE INTERNATIONAL JOURNAL OF BIOLOGICAL MACROMOLECULES)

NOPAL AS A NOVEL REINFORCING AND ANTIOXIDANT AGENT FOR STARCH BASED-FILMS: A COMPARISON WITH LIGNIN AND PROPOLIS EXTRACT

Abstract

The potential use of nopal cladode flour (NC) as reinforcing/bioactive agent in cassava starch-based films was evaluated and compared with the use of propolis extract or lignin, which are commonly employed for these purposes. Cassava starch-based films containing untreated NC (S-NC), NC treated at pH 12 (S-NC12), NC treated with aqueous propolis extract at two different concentrations (S-P1 or S-P2), or NC treated with lignin (S-L) were produced by the casting technique; glycerol was used as plasticizer. The results were analyzed by multivariate statistical analysis, which showed that NC or NC12 addition to the cassava starch matrix yielded films with distinct performance due to changes in the fiber structure and loss of phenolic compounds by alkaline treatment. Consequently, the S-NC12 film was more elongable, less crystalline, less hydrophilic, and less permeable to water vapor and displayed lower antioxidant activity by DPPH than the S-NC film. Moreover, NC12 and NC affected the mechanical properties of the cassava starch film similarly as compared to propolis extract and lignin. NC12 was better incorporated into the cassava starch matrix than lignin and propolis extract, which improved the cassava starch film hydrophobicity and antioxidant activity by ABTS. The antimicrobial activity of the films containing NC and NC12 should be further improved. Overall, nopal cladode flour has potential use in the production of active biodegradable packaging for the preservation of products with high oxidation rate.

Keywords: *Nopal (Opuntia ficus-indica), propolis, lignin, cassava starch film, antioxidant activity.*

1 Introduction

Cassava starch, which is inexpensive and easily accessible, has been extensively studied for the production of edible films (Costa, et al., 2017; Espinoza Acosta et al., 2015**;** Bher, Auras & Schvezov, 2018**).** Cassava starch films show acceptable mechanical properties but high hydrophilicity and poor active properties, which has limited their application as food packaging (Colivet & Carvalho, 2017; Edhirej et al., 2016; Zain, Kaha & Noriman, 2016). To improve these properties, many strategies have been developed to reinforce cassava starch-based films, including addition of acetylated starch nanoparticles (Teodoro et al., 2015), cassava bagasse and sugar palm fiber (Edhirej et al., 2017), piassava/lignin (Miranda et al., 2015), and soybean hulls, sugarcane bagasse and pinewood sawdust (Macías-Almazán et al., 2020). The bioactive property of cassava starch films has also been improved with addition of plant extracts, ZnO nanorods, lycopene nanocapsules, and anthocyanin (Assis et al., 2017; Estevez-Areco et al., 2020, Medina Jaramillo o et al., 2015; Qin et al., 2019).

Nopal (*Opuntia ficus-indica*) is a cactus plant that is widely used in the cosmetic and pharmaceutical industries because of its antioxidant, anticancer, and anti-inflammatory properties (Andreu et al., 2018; Ayoub et al., 2014; Bayar et al., 2016; Espino-Díaz et al., 2010; Rodriguez-Gonzalez et al., 2014; Smida et al., 2017). Given that nopal cladodes contain mucilage and fiber, they have been investigated as an interesting candidate for the development of biodegradable films and edible coatings and as a source of bioactive molecules (Allegra et al., 2017; Ayadi et al., 2009; Del-Valle et al., 2005; González Sandoval et al., 2019; Luna-Sosa et al., 2020; Sáenz, Sepúlveda & Matsuhiro, 2004; Scognamiglio et al., 2019; Treviño-Garza et al., 2017). The use of nopal cladodes is economically suitable for large-scale production, so they are an attractive alternative for the development of environmentally friendly packaging (Gheribi et al., 2018, Gheribi et al., 2019; Luna-Sosa et al., 2020; Scognamiglio et al., 2019).

Propolis is a mixture of resins, gums, balsam, waxes, essential oil, and pollen, among others, that is produced by honeybees (Oliveira et al., 2015; Suleman et al., 2015). Propolis contains large quantities of flavonoids and phenolic compounds and presents countless biological properties, such as antibacterial, antifungal, anti-inflammatory, and antioxidant activities (Nina et al., 2016; Osés et al., 2016; Socha et al., 2015). Its bioactive properties have led it to be applied in the production of edible films or coatings, in order to reduce spoilage and to extend the shelf life of fruit and vegetables (Costa et al., 2014; Khoshnevisan et al., 2019; Pastor et al., 2010; Shavisi et al., 2017; Suriyatem et al., 2018).

Lignin is a polyphenolic, amorphous, hydrophobic, low-density, and abrasive material, consisting of three phenylpropane monomeric units, coniferyl alcohol, *p*-hydroxyphenyl alcohol, and sinapyl alcohol, and it exhibits antioxidant action (Margida, Lashermes & Moorheada 2020; Shin et al., 2019). Employing lignin as reinforcer/fillers has improved the mechanical properties, antioxidant effect, and water resistance of starch-based films (Baumberger, 2002; Espinoza Acosta et al., 2015; Miranda et al., 2015**;** Shi & Li, 2016**;** Souza de Miranda et al., 2015; Spiridon et al., 2011; Yang et al., 2018; Zhao et al., 2019**)**.

Herein, the use of untreated nopal cladode flour and of nopal cladode flour treated at pH 12 as reinforcer/filler and antioxidant/antimicrobial additive in cassava starch-based films was investigated, and its performance was compared to the performance of other vegetable additives like propolis extract and lignin.

2. Methodology

2.1 Materials

Cassava starch (with 18 wt.% amylose and 82 wt.% amylopectin and 1×10^7 g/mol molecular weight) was purchased at the common market located in Ribeirão Preto (São Paulo, Brazil).

The green aqueous propolis extract from *Baccharis dracunculifolia* (Muzambinho-MG, Brazil) was furnished by Laboratory of Agroindustrial Biopolymers (LBPA) located at the Chemistry Department of Faculdade de Filosofia, Ciências e Letras in Ribeirão Preto (São Paulo, Brazil). The propolis extract contained 11% dry extract, 19.8±1.4 mg/mL total phenolic compounds (expressed as gallic acid), and 4.3±0.3 mg/mL flavonoids (expressed as quercetin).

Glycerol and the radical scavengers DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) and ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) were supplied by Sigma-Aldrich (São Paulo, Brazil). Sodium hydroxide P.A was acquired from Dinâmica – Química Contemporânea (São Paulo, Brazil).

2.2 Lignin production and NMR analyses

Lignin was extracted from sugarcane bagasse by the organosolv method with slightly acidic ethanol, as described by Jääskeläinen et al. (2017). In short, the bagasse was mixed with the extraction solution at a 10:1 (w/w) ratio in a 2-L Parr reactor (serie 4530, USA) and heated at 110 °C. After reaction for 1 h, the liquid and solid phases were separated by vacuum filtration, and lignin was precipitated with water, centrifuged, and freeze-dried for 12 h in an Alpha LSC Basic 1-4 (Christ, Germany).

Lignin was characterized by 1H-13C HSQC NMR spectra (heteronuclear single-quantum coherence, HSQC) in an ADVANCE DRX 400 MHz (Bruker, Karlsruhe, Germany) at the NMR facility of the Federal University of Minas Gerais (UFMG). For the NMR of isolated lignin, 40 mg of the sample was dissolved in 0.75 cm^3 of DMSO-d_6 and 0.25 cm^3 of pyridine-d_5. HSQC were assigned by comparison with the literature (Del Rio et al., 2012a), and the detailed NMR experimental conditions can be found elsewhere (Del Rio et al., 2012b).

2.3 Nopal cladode flour characterization and production

The nopal cladodes (*Opuntia ficus-indica)* were freshly harvested in the Northeastern region of Brazil (3.7327° S, 38.5270° W) at a plantation belonging to the Animal Science Department of the Federal University of Ceará (Ceará, Brazil). From these harvested cladodes, spineless cladodes were cut into cubes, dried in an oven with forced air circulation (60 °C/12 h) (Quimis, Q314M292, Brazil), and grounded and sieved through 120-mesh sieve, to obtain the nopal cladode flour.

The moisture (AOAC 920.151, 1997), ash (AOAC 923.03, 1997), lipid (Bligh & Dyer, 1959), and protein (AOAC 926.86, 2005) contents of the nopal cladode flour were analyzed, in triplicate.

2.4 Nopal cladode flour chemical treatment

The nopal cladode flour (NC) was pretreated before it was used to prepare cassava starch-based films. To this end, 5 g of NC were dispersed in 95 g of distilled water and homogenized with a homogenizer at 10.000 rpm at room temperature for 4 min (Tecnal, TE-102 Turratec). Then, the pH was adjusted to 12 with 0.1 M NaOH solution. NC dispersion at native pH (4.5) was also prepared. Both dispersions were maintained at room temperature (~25 °C) and kept in dark containers for 12 h, followed by filtration (150- mesh sieve), to separate the largest particles that could impair the formation of a homogenous film. The filtered solids were separated, and the supernatant containing 2.75 g of NC was used to prepare the composite films.

2.5 Film production

Cassava starch-based films without (control) and with added NC, NC12, lignin, or propolis extract were prepared. Briefly, 100 g of filmogenic solution was obtained by using 4 g of cassava starch and 1 g of glycerol for all the films. Next, 95 g of the supernatant

containing 2.75 g of NC or NC12 was employed to prepare the S-NC and S-NC12 films, whereas 0.4 g of lignin and 94.6 g of distilled water were added to prepare the S-L film. Films with propolis were prepared by incorporating 0.6 g of aqueous propolis extract (0.066 g of dry extract) and 94.4 g of distilled water into the S-P1 film, and 1.2 g of propolis (0.132 g of dry extract) and 93.8 g of distilled into the S-P2 film. The lignin and propolis concentrations were established from preliminary tests (data not shown). All the suspensions were heated at 85 ± 1 °C until a semi-viscous solution emerged. The dispersions were individually cast onto an acrylic plate (0.155 g per cm^2) and dried in an oven (BOD, Marconi) at 32 °C for 10 h. The dried films were conditioned in a desiccator at 25 °C and 53% relative humidity for 48 h before they were analyzed.

2.6 Film thickness and mechanical properties

Film thickness was measured with a Zaas-Precision flat tip digital micrometer (1-μm resolution).

The mechanical tests were conducted with a texture analyzer TA TX Plus (TA Instrument, England). Tensile strength (TS) and elongation at break (E) were obtained according to the ASTM Method D882-12 (2012) by taking an average of ten determinations in each case. The films were cut into 2.54-cm-wide strips measuring at least 10 cm. The initial grip separation was 80 mm, and the crosshead speed was 1.0 mm s^{-1}. Young's modulus (MY) was calculated as the inclination of the initial linear portion of the stress versus strain curve by using the software Texture Expert V.1.22 (SMS). All the measurements were performed in triplicate.

2.7 Film opacity

Film opacity was determined by measuring the absorbance at 600 nm on a spectrophotometer (HP Hewlett Packard 8453, USA). Each film was cut into a rectangular

piece and directly placed into the spectrophotometer test cell. An empty test cell was used as reference. Opacity was calculated by the following equation (Liu et al., 2017):

$$\text{Opacity} = A/x \quad , \qquad (1)$$

where A = absorbance at 600 nm and x = film thickness (mm). Measurements were performed in triplicate.

2.8 Film functional properties

Film solubility in water, moisture content, contact angle, and water vapor permeability (WVP) were analyzed in triplicate.

Film solubility in water was calculated as the percentage of dry matter of the solubilized films (discs with diameter of 20 mm) after immersion in 50 mL of water at 25 ± 2 °C for 24 h (Gontard, Guilbert & Cuq, 1992). The film moisture content was determined by the ASTM D644-07 Standard method (ASTM, 2007). The film contact angle was measured according to the ASTM Method D7334–08 (ASTM, 2013); OCA-20 Dataphysics (OCA 20, Dataphysics, Germany) was used. Water drop (surface tension = 72.7 mN/m) images were taken at room temperature and in air every 2 min. The image processing software GIMP 2.6.8 was used to measure the contact angle of the surface exposed to drying, which was identified as the tangent to the drop edge in the intersection of the liquid, solid, and gaseous media.

The film WVP was gravimetrically measured according to the ASTM Method E96/E96M (ASTM, 2014) at 25 ± 2 °C. The film samples were sealed over the circular opening of a permeation cell containing silica gel, and the cells were placed in desiccators containing distilled water (steady-state condition). The cell was weighed every 1 h, for 9 h, on an analytical scale. The film WVP was calculated as $WVP = w.x/t.A.\Delta P$, where "x" is the average film thickness, "A" is the film permeation area (0.00196 m^2), and "ΔP" is the difference between the partial pressure of the atmosphere over silica gel and over pure water

(3.168 kPa, at 25 °C); the term w/t was calculated by linear regression on the basis of the weight gain data as a function of time.

2.9 X-ray diffraction analysis

X-ray diffraction analyses of the films were performed on a Siemens X-ray diffractometer (Siemens, model D5005, Baden-Württemberg, Germany) operating at 40 kV and 30 mA with Kα copper radiation and monochromatic filter at room temperature. The data were collected from 2 to 50 °C on a 2θ scale at a rate of 0.02° / s. The crystallinity index (%) of the materials was quantitatively estimated as the ratio of the crystalline area of the individual peaks by the total area of the total diffractogram area; the Nara & Komiya (1983) method was followed, and the software Origin 8.1 was used (OriginPro Corporation, Massachusetts, USA).

2.10 Total phenolic compounds quantification and antioxidant activity

To determine the total phenolic compounds content and the antioxidant activity by DPPH and ABTS, deionized water was use as solvent to prepare the extract. Total phenolic compounds were determined according to Larrauri et al. (1997), with some modifications.

The antioxidant activity was determined by the DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) (Martins et al., 2012) and the ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) methods (Rufino et al., 2007). All the analyses were performed in triplicate.

2.11 Film antimicrobial activity

Film antimicrobial activity was evaluated by the agar diffusion assay according to Pelissari et al. (2009) and Shapi'i et al. (2020), with some modifications; four microorganisms were employed: *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans.* Film discs with diameter of ~1.5 cm were sterilized under UV light for 8

min on each side before the antimicrobial test was conducted. Each film disc was aseptically placed on the surface of the previously inoculated Tryptic Soy Agar (TSA) culture medium with 100 µL of suspension (MacFarland scale 1) of each microorganism. The plates were incubated at 37 °C for 24 h. The test was carried out in duplicate for each sample, on two separate occasions.

2.12 Multivariate statistical analysis of the films dataset

A numerical matrix was constructed by using the dataset of the control, S-NC, S-NC12, S-P1, S-P2, and S-L film properties, which included the moisture content, solubility in water, opacity, WVP, contact angle, mechanical properties, total phenolic compounds content, and antioxidant activity determined by DPPH and ABTS. The resulting matrix consisted of 18 film samples (triplicate of six types of starch-based films) and 11 variables (film properties) and was imported with the software PLS Toolbox™ (version 8.6.2, Eigenvector Research Incorporated, Manson, WA USA). An unsupervised multivariate statistical evaluation by Principal Component Analysis (PCA) was developed to explore sample variability according to their parameters. Before the chemometric approach, the film data were autoscaled (mean centered with subsequent variance scaling to unit), which provided the same response strength for all the variables in the modeling. The Singular Value Decomposition (SVD) algorithm was applied to decompose the numerical matrix into score and loading systems, as well as modeling errors and sample influences (Maria de Fátima et al., 2020). The relevant information about film variance was obtained on the first two Principal Components (PC1 and PC2) under a confidence level of 95%.

2.13 Univariate statistical analysis

From the collected data on film properties an analysis of variance (ANOVA) was conducted at a 95% confidence interval; the software Statistica 7.0 (Statsoft$^{@}$) was employed.

Ducan's test was performed here to evaluate better the average differences ($p < 0.05$) of properties between the films produced in this work.

3. Results and discussion

3.1 Lignin structural analysis

The extracted lignin was structurally analyzed by 1H-13C HSQC NMR analysis. Figure 1a shows some carbohydrate signals in the aliphatic side-chain region; these signals overlapped with some lignin signals. The intense lignin correlation signals corresponding to the Cα/Hα and Cβ/Hβ of β-O-4 substructures (A, A') emerged in the δC/δH range 57.8–88.5/3.42–5.10, respectively. The phenylcoumaran substructure (B) was also detected by the HSQC analysis, albeit at low intensity.

In the aromatic/unsaturated region shown in Figure 1b, one can see the correlations between the lignin units G and S and the end-group structures *p*-hydroxycinnamates (PCA) and ferulates (FA), which are typically detected in grass-type lignin like sugarcane (Del Rio et al., 2015). The absence of strong carboxylic group correlation signals demonstrated that the mild extracting conditions did not promote further oxidation or etherification reactions within the lignin structure.

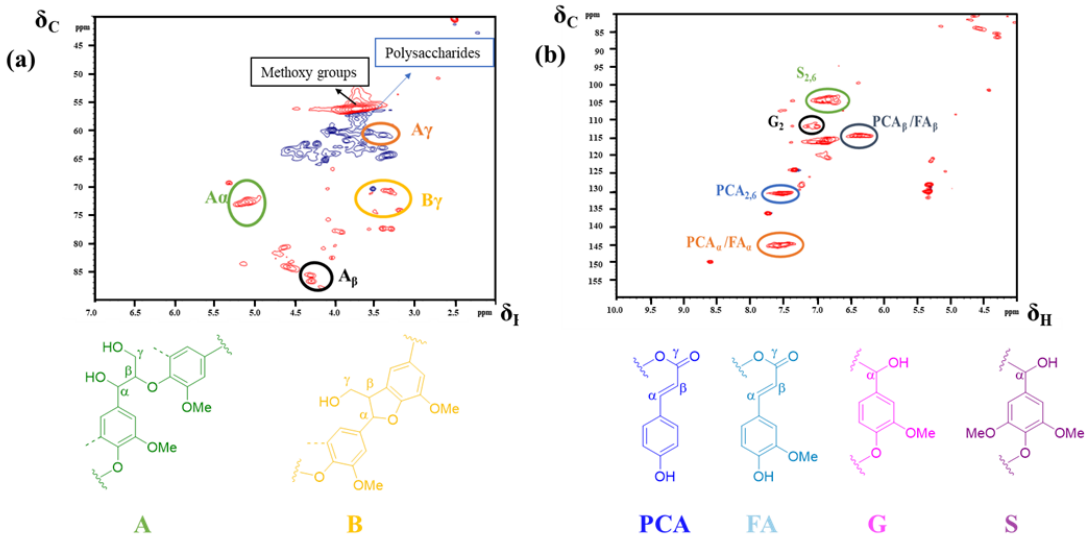

Figure 1. Structural components detected in sugarcane lignin by 1H-13C HSQC NMR.

3.2. Nopal cladode flour chemical composition

The nopal cladode flour-120 mesh (NC) contained 10.9 ± 0.79 % of moisture, 21.4 ± 0.16 % of ash, 2.9 ± 0.37 % of lipids, 3.3 ± 0.10 % of protein, and 72.4 ± 0.60 % of carbohydrates. Some authors reported similar protein content (Du Toit, De Wit & Hugo, 2018, Du Toit et al., 2019), whereas others found higher protein (8.8%) and ash (24%) contents in nopal cladodes (Yang et al., 2015). These differences could be due to variations in nopal plant cultivation conditions such as soil, climate, and region. Regarding the NC bioactivity, the total phenolic compounds content was 13.49 ± 0.45 mg of GAE/g of NC, and the antioxidant activity determined by the DPPH and ABTS assays was 87.49 ± 0.66 % and 20.93 ± 0.20 μM Troloxg^{-1}of NC, respectively. Thus, this flour fraction was chosen, as demonstrated in Paper 1 (Nopal cladode flour: production, characterization, and its evaluation for producing edible film), to be used as reinforcer/filler and antioxidant/antimicrobial additive in cassava starch-based films formulation.

3.3 Multivariate statistical analysis of the film mechanical and functional properties

A multivariate statistical analysis by PCA was performed to reduce the dimensionality of the multivariate data while preserving most of the variance and to observe the natural "patterns" in a dataset (Wold, Esbensen & Geladi, 1987). The patterns were visualized by calculating principal components, whose function is to maximize the variance and covariance of a dataset and which can be divided into a complementary set of scores and loadings (Wold, Esbensen & Geladi, 1987).

Figure 2 presents the scores (A) and loadings (B) plots of the film dataset (six type of films produced in triplicate × 11 responses or film properties) along principal components (PC) 1 and 2: cassava starch films (control), cassava starch-based films with added NC (S-NC) and NC pretreated at pH 12 (S-NC12); cassava starch-based films

containing 0.6 g (S-P1) and 1.2 g (S-P2) of propolis; and cassava starch-based film containing lignin (S-L). Figure 2Aa illustrates the PC1 × PC2 scores from the evaluation of all the aforementioned films together; Figure 2Ab presents the PC1 × PC2 scores from the evaluation of all the films without the control film, to highlight differences among the S-NC, S-NC12, S-P1, S-P2, and S-L films; and Figure 2Ac presents the PC1 × PC2 scores from the evaluation of the S-NC and S-NC12 films only, to highlight the effect of the pretreatment at pH 12. Figure 2B illustrates the respective bidimensional loading plots.

Figure 2Aa shows that the PC1 axis retained the most relevant variance from the influence of NC (NC and S-NC12 at positive score), propolis extract, or lignin (S-P1, S-P2, and S-L at null scores) addition to the cassava starch film (control film at negative scores). Because the S-NC and S-NC12 films presented opposite scores related to the PC1 axis as compared to the cassava starch film (control), NC or NC12 addition impacted the properties of the cassava starch film to a larger extent than propolis extract or lignin addition. Moreover, the S-NC and S-NC12 films had higher coefficient on PC1 than the S-P1, S-P2, and S-L films, which demonstrated their importance for the total variability of the analyzed data. The PC2 axis in Fig. 2Aa also showed relevant information to differentiate the S-NC and S-NC12 films from the films containing propolis (S-P1 or SP2) or lignin (S-L), which were clearly discriminated in Figures 2Ab and 2Bb.

On the basis of Figure 2Ba, thickness, total phenolic compounds content, antioxidant activity determined by ABTS and DPPH, and opacity were the properties that were the most affected by NC, NC12, propolis, or lignin addition to the cassava starch film, which indicated that addition of these agents improved these properties. The PC2 axis pointed out that the contact angle and YM were also slightly affected by NC, NC12, propolis, or lignin addition to the cassava starch film.

The score results illustrated in Figure 2Ab evidenced that the S-P1 and S-P2 films were situated close to one another in the PC1 and PC2 axes, indicating that the propolis concentration used in this work little influenced the film properties. According to Figure 2Bb, propolis addition to the cassava starch film produced films with high tensile strength (TS) and Young's modulus (YM), whereas lignin addition caused more variability in opacity, contact angle, and elongation at break (EB). Furthermore, Figure 2Ab revealed that the S-NC and S-NC12 films were situated far apart from the S-P1, S-P2, and S-L films along the PC1 axis, showing higher values of solubility, total phenolic compounds content, antioxidant activity determined by DPPH and ABTS, WVP, thickness, and moisture content than the other films. Other relevant information provided by the multivariate analysis was the difference between the properties of the S-NC and S-NC12 films as shown in Figure 2Ac. The NC alkaline treatment increased the solubility, moisture content, antioxidant activity determined by ABTS, and contact angle of the S-NC12 films (Figure 2Bc).

Therefore, after the trend in the film parameters concerning their composition achieved by multivariate statistical analysis was understood, univariate statistical analysis (analysis of variance) was conducted to reveal the differences in the properties of the six types of films produced herein. The results are detailed in the following subsections.

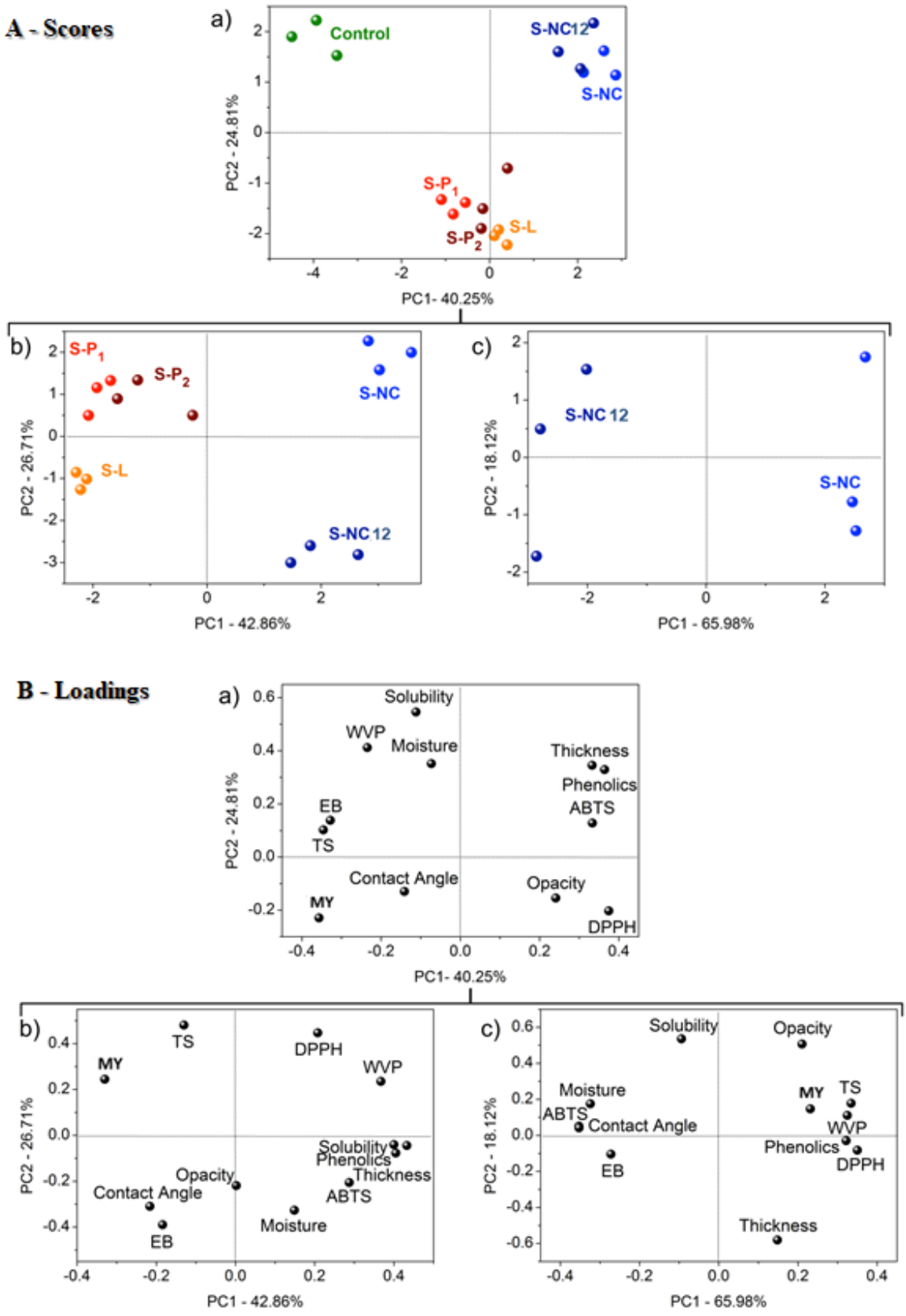

Figure 2. (**A) -** Scores coordinate systems (PC1 × PC2) from starch-based films with different compositions: a) control, S-NC, S-NC12, S-P1, S-P2, and S-L films; b) S-NC, S-NC12, S-P1, S-P2, and S-L films; and c) S-NC and S-NC12 films. **Fig. 2. (B)**- Loadings (PC1 × PC2) from control, S-NC, S-NC12, S-P1, S-P2, and S-L films; b) S-NC, S-NC12, S-P1, S-P2, and S-L films; and c) S-NC and S-NC12 films.

3.3 Film visual aspect and opacity

Among the films produced in this work, the S-NC and S-NC12 films were easier to handle and more stable to the surrounding environment. Meanwhile, the S-L film presented some visible lignin particles on the film surface, a result of its low solubility in water and the low degree of compatibility between lignin and the starch matrix (Zhao et al., 2019). The other films had a more homogenous aspect, which suggested better incorporation of NC, NC12, or propolis into the cassava starch matrix, as can be seen in Figure 3, which also depicts the opacity of the control, S-NC, S-NC12, S-P1, S-P2, and S-L films. All the films allowed dots printed on the paper to be observed, which attested to their transparency. However, the films presented different color. The cassava starch film was colorless and more transparent, whilst NC, NC12, propolis, or lignin incorporation into the cassava starch matrix provided a colorful film. Among the agents used in this work, lignin incorporation into the cassava starch matrix yielded the darkest film, which was brownish, whereas propolis afforded the least colorful films. On the other hand, NC or NC12 incorporation produced films with different colors: the S-NC film was slightly brownish, while the S-NC12 film was greenish.

As for film opacity, the cassava starch film (control) was the least opaque (3.5 A_{600}/mm), whereas NC, NC12, P2, or L incorporation into the cassava starch matrix significantly increased film opacity ($p < 0.05$). P1 addition did not affect the cassava starch film opacity because the propolis concentration was low. The S-L film was the opaquest (S-L: 23.7 A_{600}/mm), followed by the S-NC (13.6 A_{600}/mm) and S-NC12 (12.6 A_{600}/mm) films. In contrast, the S-NC and S-NC12 film opacity did not differ significantly. The S-NC and S-NC12 film opacity differed from the values reported by González Sandoval et al. (2019) (~7.4) and Lira-Vargas et al. (2014) (~ 2.94)—these authors used nopal mucilage to prepare the films, whilst the nopal cladode flour (integral material) was employed herein. About the

S-L film, Michelin et al. (2020) reported a similar opacity and a brownish color for lignin films added with CMC (carboxymethyl cellulose).

Opacity is a desirable characteristic for packaging of light-sensitive food because exposure to light can accelerate food degradation, thereby affecting food taste, freshness, and nutritional value (Liu et al., 2017; Pacheco et al., 2019, Rao et al., 2010; Riga et al., 2019). Therefore, NC, NC12, and lignin can be used to improve the light barrier of cassava starch film, to obtain packaging for light-sensitive food.

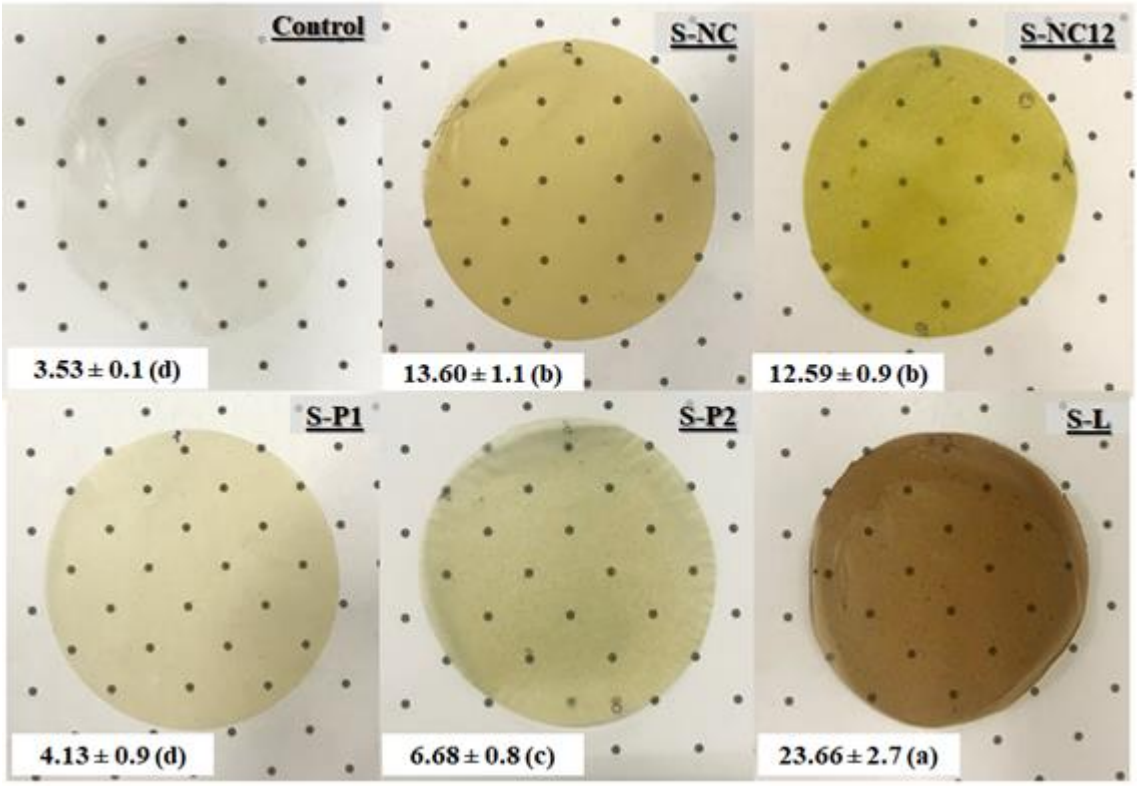

Figure 3. Visual aspect and opacity values of cassava starch-based films without (control) and with addition of untreated nopal cladode flour (S-NC), treated nopal cladode flour (S-NC12), aqueous propolis extract (S-P1 or S-P2), and lignin (S-L). Opacity is expressed in A_{600}/mm. The different letters in parenthesis indicate a statistically significant difference according to Duncan's test ($p < 0.05$).

3.4 Thickness and mechanical properties.

Propolis extract (P1 or P2) or lignin incorporation into the cassava starch film did not affect film thickness, as shown in Table 1. Nonetheless, NC or NC12 addition increased the cassava starch film thickness by around 68.75% (S-NC – 0.087 mm and S-NC12 – 0.080 mm), which can be justified by the increase in the solid content and the presence of fibers in NC. The S-NC and S-NC12 films had similar thickness, which evidenced that the chemical treatment did not reduce the solid content or significantly damaged the fibers present in NC.

Table 1 also lists the mechanical properties of the control, S-NC, S-NC12, S-P1, S-P2, and S-L films. The control film was slightly more mechanically resistant and elongable than the S-NC, S-NC12, S-P2, and S-L films. Meanwhile, the cassava starch and the S-P1 films had similar tensile strength (TS) and Young's modulus (YM) values, but addition of even low P concentration to the cassava starch matrix (S-P1) decreased the film elongation at break (EB). Mustafa et al. (2020) and Villalobos et al. (2017) also reported that addition of low concentration of propolis extract to starch-based films impacted the TS property little due to a good interaction between the cross-linked starch and propolis.

Table 1. Thickness, tensile strength (TS), elongation at break (EB), and Young´s modulus (YM) of pure cassava starch film (control) and cassava starch-based films added with NC (S-NC), NC12 (S-NC12), aqueous propolis extract (S-P1 or S-P2), and lignin (S-L).

Film	Thickness(mm)	TS (MPa)	E (%)	YM (MPa)
Control	0.053 ± 0.01^{b}	3.80 ± 0.80^{a}	44.69 ± 4.33^{a}	147.26 ± 2.91^{a}
S-NC	0.087 ± 0.09^{a}	$3.00 \pm 0.18^{b,c}$	18.93 ± 0.58^{c}	$81.60 \pm 0.04^{b,c}$
S-NC12	0.080 ± 0.07^{a}	2.47 ± 0.48^{c}	$28.46 \pm 6.49^{b,c}$	71.17 ± 0.12^{c}
S-P1	0.051 ± 0.03^{b}	$3.25 \pm 0.40^{a,b}$	22.62 ± 5.07^{c}	146.10 ± 0.26^{a}
S-P2	0.050 ± 0.07^{b}	$2.99 \pm 0.40^{b,c}$	22.78 ± 1.35^{c}	142.40 ± 0.28^{a}
S-L	0.056 ± 0.04^{b}	$2.72 \pm 0.53^{b,c}$	34.14 ± 1.96^{b}	105.87 ± 0.0^{b}

a, b, c, d: Mean values in the same column with different lowercase letters are significantly different ($p < 0.05$) as revealed by Duncan's test, $p < 0.05$.

Nevertheless, NC or NC12 incorporation into the cassava starch film had a different effect on the film mechanical properties. NC addition decreased the cassava starch film TS, EB, and YM values by 21%, 57.7%, and 44.6%, respectively, whereas NC12

addition reduced the TS and YM values (35% and 51.7%), but it had a weaker effect on the film EB (36.3%). NC contains fiber and mucilage, which are large molecular structures that can form a disordered structure in the cassava starch matrix, to impact the starch-starch interactions and, consequently, the film mechanical properties. Films based on nopal mucilage have also been shown to have low mechanical resistance (Espino-Díaz et al., 2010, González Sandoval et al., 2019; Jacquot et al., 2014; Lira-Vargas et al., 2014). Comparison between the S-NC and S-NC12 films showed that the use of NC12 provided a slightly more elongable, less rigid, and less mechanically resistant film than the use of NC. The major S-NC12 elongation could be explained by the presence of sugars resulting from the NC12 chemical treatment of NC12: these sugars can act as plasticizer, thus increasing film flexibility (González Sandoval et al., 2019).

On the other hand, lignin addition to the cassava starch film, to give the S-L film, decreased the TS, EB, and YM values by 28.4%, 23.6%, and 28.1%, respectively. Espinoza Acosta et al. (2015) also observed that lignin incorporation into a durum wheat starch matrix decreased the film TS and YM, while EB increased. Similar results were achieved by Shi & Li (2016), who studied starch/lignin films, and by Zhao et al. (2019), who investigated lignin addition into cellulose nanofiber/starch composite films. However, lignin (6%) addition improved the mechanical strength (from 14.5 to 23.1 MPa) of a biopolymeric soy protein plastic sheet, but high lignin concentration decreased the TS values (Chen et al., 2006). In this sense, the concentration of 10% used herein may have afforded a lower degree of compatibility between starch and lignin, to result in the S-L film having a low TS value (Baumberger, Lapierre & Monties, 1998a; Baumberger, Lapierre & Monties, 1998b). As mentioned previously, the S-L film contained some lignin particles that had not completely dissolved into the starch matrix.

3.5. Moisture, water solubility, and water vapor permeability (WVP)

Table 2 summarizes the properties related to the water resistance of the cassava starch film (control) and the S-NC, S-NC12, S-P1, S-P2, and S-L films. The control film presented the highest moisture content (15.2%). NC, P1, P2, or lignin incorporation decreased the cassava starch-based film moisture, whereas NC12 incorporation did not affect the moisture content. The use of NC or lignin significantly decreased the cassava starch film moisture content, yielding less humid films (S-NC: 11.76%, S-L: 11.37%). This suggested that, in the presence of these agents, a smaller number of -OH groups in the films were available to interact with the water molecules (Arezoo et al., 2020; Merino et al, 2018). Lignin can interact with free hydrophilic groups in the matrix via hydrogen interaction, diminishing water molecule adhesion and affecting how films with lignin absorb water molecules (Aadil, Prajapati & Jha, 2016; Michelin et al., 2020).

The higher S-NC12 moisture content as compared to the S-NC film suggested the presence of other compounds such as sugar in NC12 due to the chemical treatment at pH 12. These compounds contributed with the hydroxyl groups in the S-NC12 film. The higher S-NC12 moisture could explain the major plasticization (>E) and a reduction in rigidity (<MY) when compared to the S-NC film.

In the case of film solubility, Table 2 shows that the control film was the most soluble, whereas NC, NC12, P1, P2, or lignin incorporation into the cassava starch matrix decreased the film solubility in water ($p < 0.05$). Lignin (S-L film) reduced the cassava starch film solubility the most effectively (39%), whereas NC and NC12 were the least effective (~12.4%). Michelin et al (2020) and Bhat et al. (2013) also reported low solubility for a CMC/lignin film and sago starch/lignin films, respectively. Lignin has hydrophobic character, which may explain the low solubility of films incorporated with it. Furthermore, the presence of some compounds such as waxes and fatty acids in propolis extract may lower the solubility of the S-P1 and S-P2 films (Eskandarinia et al., 2019). The use of NC as additive in the

cassava starch film elicited an intriguing behavior: NC addition yielded the S-NC film, with the lowest moisture content, but this film was more soluble than the S-P1, S-P2, and S-L films, and its solubility resembled the solubility of the S-NC12 film. This suggested that the S-NC film had a more hydrophilic character, which was verified by its smallest contact angle value (26.9º, Figure 4). Therefore, the S-NC film was the most wettable film produced herein, whilst the S-NC12 film was the least wettable (contact angle = 70.85º). The presence of polar groups in the mucilage that was present in the untreated NC could account for the major hydrophilic character of the S-NC film. Other authors observed reduced contact angle of pectin-based films when nopal mucilage powder was added (Luna-Sosa et al., 2020). On the other hand, the alkaline treatment of the nopal cladodes may have modified the external surface of the fiber cell wall, thereby depolymerizing cellulose and exposing the short crystallites (Li, Tabil & Panigrahi, 2007), which caused the S-NC12 film surface to be more hydrophobic. Meanwhile, addition even small propolis concentrations significantly affected the composite film water contact angle. The contact angle increased from 57.6 ° (control) to 65.44° (S-P1) and 67.33 °(S-P2) when propolis extract was added to the cassava starch matrix. Eskandarinia et al. (2019) also reported that propolis addition to corn starch-based films contributed with the hydrophobic surface by increasing the contact angle from 47.38 ° to 73.45°. In contrast, lignin incorporation slightly decreased the composite film contact angle (53.08 °), also observed by Shankar, Reddy & Rhim, (2015) for agar/lignin films, which were more hydrophilic than the agar film. These authors reported that the poor miscibility between the polymeric matrix and lignin caused irregularities on the lignin film surface, thus affecting the attractive forces between the water drop and the film surface and reducing the contact angle.

NC, NC12, propolis extract (P1 or P2), or lignin addition to the cassava starch film decreased the film water vapor permeability (WVP). In other words, all the agents used herein improved the cassava starch film water vapor barrier. Moreover, as observed in the

case of film solubility, the S-NC film also had greater WVP than the S-L, S-P1, S-P2, and S-NC12 films. Thus, NC addition may have created pores on S-NC film surface, which allowed water molecules to diffuse through the film. On the other hand, NC12 may have been better incorporated in the cassava starch matrix, to yield a film that was less permeable to water vapor than the S-NC film. The S-NC12 film and nopal mucilage-based film had similar WVP (7.6 x 10^{-11} and 8.40 × 10^{-11} g·m^{-1}·s^{-1}·Pa^{-1}, respectively) (González Sandoval et al., 2019). Because WVP is influenced by the internal structure of each film (Guadarrama-Lezama et al., 2018), the S-NC12, S-P1, S-P2, and S-L films presented a more compact internal structure than the S-NC and control films, which reduced water permeation. Besides that, the presence of hydrophobic compounds in propolis extract and lignin may also have reduced film WVP as compared to the control film. In CMC/lignin-based films and agar/lignin films, lignin addition also diminished WVP (Michelin et al., 2020, Shankar, Reddy & Rhim, 2015). According to these authors, the lignin hydrophobic character modified the polymeric matrix barrier by increasing the tortuous path for water vapor diffusion, which explains the low water permeation through these films.

Table 2. Moisture content, solubility, water vapor permeability (WVP), and opacity of cassava starch film without (control) and with NC (S-NC), NC12 (S-NC12), aqueous propolis extract (P1 and P2), and lignin (S-L).

Film	Moisture (%)	Solubility (%)	WVP*
Control	15.2 ± 0.30^{a,b}	50.25 ± 1.00^{a}	1.42 ± 0.07^{a}
S-NC	11.76 ± 0.25^{c,d}	43.62 ± 1.69^{b}	1.03 ± 0.01^{b}
S-NC12	15.08 ± 1.26^{a}	44.60 ± 2.05^{b}	0.76 ± 0.01^{c}
S-P1	12.78 ± 0.63^{b,c}	35.81 ± 2.23^{c}	0.68 ± 0.01^{c}
S-P2	12.61 ± 0.10^{c}	36.31 ± 2.53^{c}	0.72 ± 0.01^{c}
S-L	11.37 ± 0.63^{d}	30.55 ± 1.19^{d}	0.68 ± 0.03^{c}

Values with different lowercase letters in the same column are significantly different ($p < 0.05$).
WVP: Water Vapor Permeability (10^{-10} g.m^{-1}.s^{-1}Pa^{-1}).

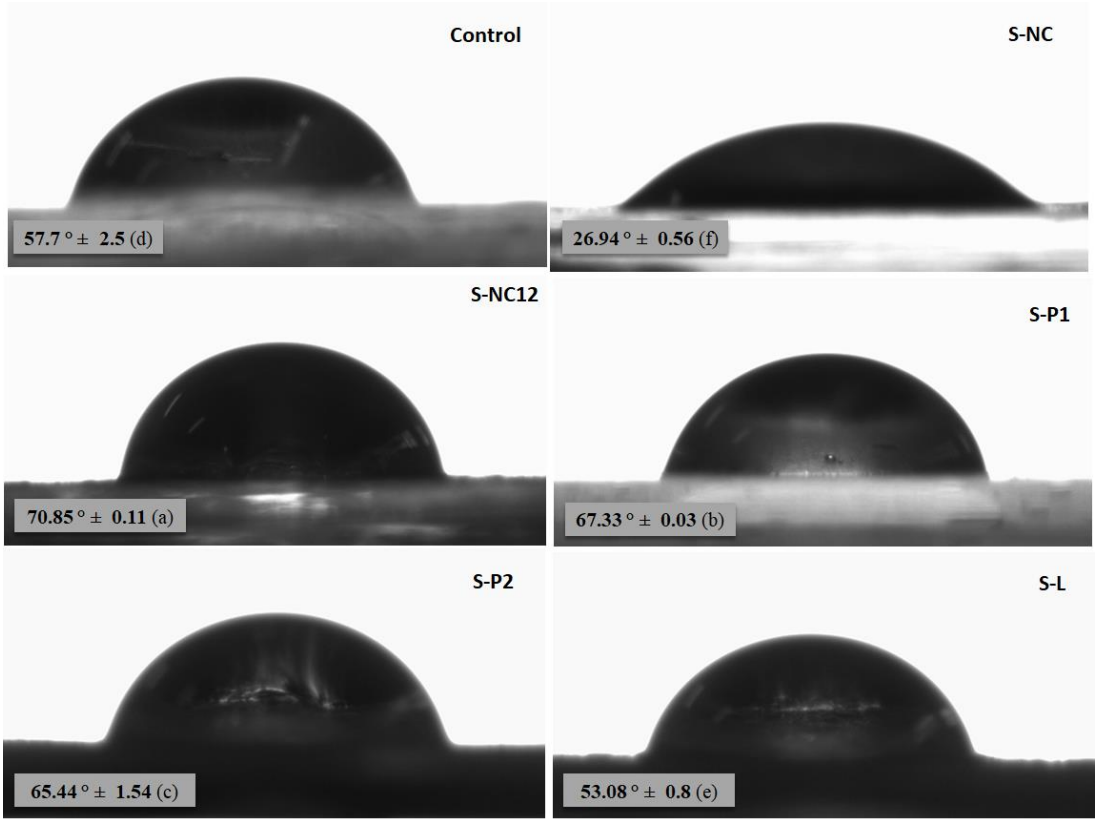

Figure 4. Water drop deposited on the film surface the contact angle degree (θ) of pure cassava starch film (control) and cassava starch-based films added with NC (S-NC), NC12 (S-NC12), aqueous propolis extract (S-P1 or S-P2), or lignin (S-L). Values with different lowercase letters between parentheses are significantly different ($p < 0.05$).

3.6 X-ray diffraction (XRD)

The diffractograms presented in Figure 5 evidenced that the control film had one peak between 2θ = 19° and 23º, which corresponded to an amorphous phase B-type pattern and V-type crystalline structure due to interaction between glycerol and the amylose helix, in agreement with others findings for the cassava starch film (Chang-Bravo, López-Córdoba & Martino, 2014; Medina-Jaramillo, Bernal & Famá, 2020). L, P1, or P2 incorporation did not affect the control film diffractogram, whereas NC and NC12 addition changed the diffractogram as compared to the control film, with the presence of peaks at 2θ = 15º, 25º, 30º, and 38º. The peaks at 2θ = 15°, 25° and 38° were related to the presence of calcium oxalates ($C_2CaO_4H_2O$), which is the major crystalline component in nopal powders. The peak

at 2θ = 30° referred to calcium carbonate as calcite ($CaCO_3$) and to calcium hydride (CaH_2), while the small peak at 2θ = 36° was attributed to potassium peroxydiphosphate ($K_4P_2O_8$) (Contreras-Padilla et al., 2011; Luna-Sosa et al., 2020; Madera-Santana et al., 2018). The presence of calcium carbonate in the film structure can be advantageous because it provides the film with additional protection, promoting artificial shading for the packaged product and consequently protecting it from light, reducing stress, and regulating gas exchange (Da Silva et al., 2019; Saeb et al., 2013). Although the S-NC and S-NC12 films displayed similar diffractograms, the latter film was less crystalline (19.4%) than the S-NC film (23.2%), which suggested that the alkaline treatment applied to NC modified its fiber structure and generated more amorphous zones in the S-NC12 film. The low crystallinity of the S-NC12 film may underlie its higher elongation at break as compared to the S-NC film (Table 1).

In addition, when it comes to the highest crystallinity value shown by the S-P2 film (27%), Mustafa et al. (2020) and Villalobos et al. (2017) explained that, at low concentration, propolis extract interacts well with the starch matrix; however, when the propolis concentration increases, crystalline structures emerge between cross-linked starch and propolis

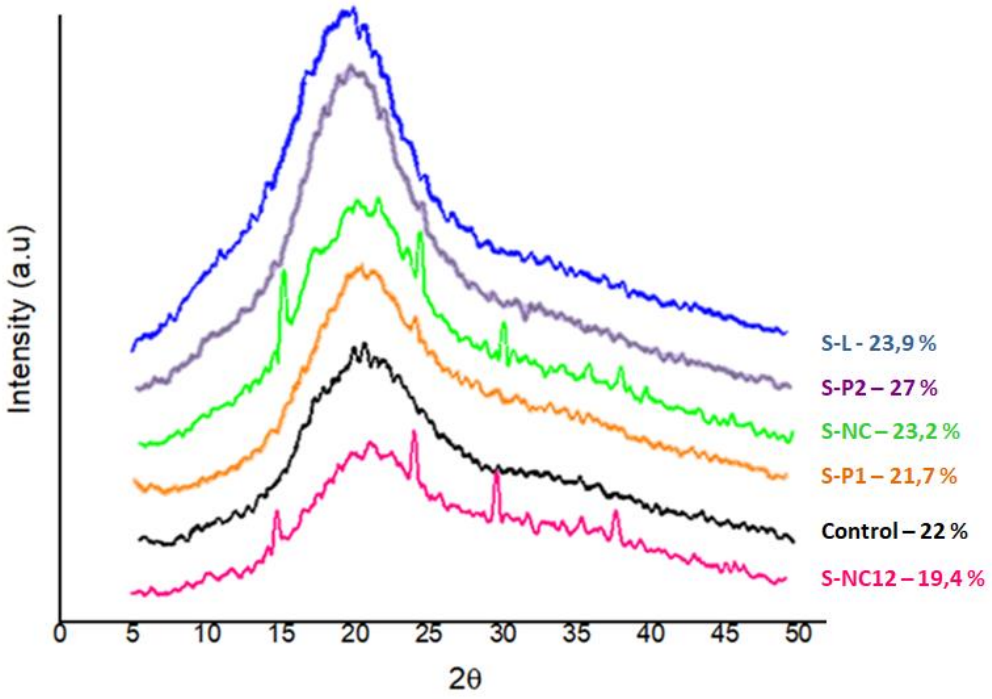

Figure 5. X-ray diffraction and percentage of crystallinity of the pure cassava starch film (control) and the cassava starch-based films added with NC (S-NC), NC12 (S-NC12), propolis aqueous extract (S-P1 or S-P2), or lignin (S-L).

3.7 Total phenolic compounds content and antioxidant capacity

Table 3 lists the total phenolic compounds content and the antioxidant capacity determined by the DPPH and ABTS methods of the control cassava starch film and the cassava starch films added with NC, NC12, P1, P2, or lignin. The control film did not display antioxidant activity due to its insignificant total phenolic compounds content (1.58 mg of GAE/g of film). NC and NC12 addition considerably contributed to the total phenolic compounds content of the cassava starch film when compared to propolis extract and lignin: the content increased from 1.58 to 21.9 (S-NC) and 18.5 (S-NC12) mg of GAE/g of film. Thus, NC and NC12 addition furnished films with greater antioxidant activity as measured by the DPPH and ABTS assays. However, Table 2 shows that the S-NC film had the best antioxidant activity according to the DPPH assay, while the S-NC12 film had the highest antioxidant activity as measured by the ABTS assay. Although the $ABTS^{\bullet+}$ and DPPH assays

are based on the same radical scavenging principle, the ABTS method evaluates lipophilic and hydrophilic antioxidant compounds, whilst the DPPH method can only measure compounds of hydrophilic nature (Dastmalchi et al., 2011; Maniglia & Tapia-Blácido, 2019). In this sense, the S-NC12 film could contain more lipophilic compounds that were solubilized during the alkaline treatment. The presence of these compounds can also justify the hydrophobic S-NC12 film surface, as observed by means of the contact angle measurement (Table 2).

In contrast, propolis addition even at low concentrations afforded films with greater antioxidant capacity than the film added with lignin (S-L). Despite the low phenolic compounds content in the S-L film, this film presented antioxidant activity measured by DPPH, which could be attributed to some lignin groups, such as monomers and oligomers that are present in the films (Crouvisier-Urion et al., 2016). Other authors reported that lignin addition at low concentration did not improve the antioxidant capacity of gelatin-based and CMC-based films, but high lignin concentrations, around 40 and 50%, yielded films with 67.51% to 83.75% antioxidant activity as measured by DPPH (Aadil, Barapatre & Jha, 2016, Michelin et al.,2020).

Table 3. Total phenolic compounds content and radical scavenging activity by DPPH and ABTS of pure cassava starch film (control) and cassava starch-based films added with NC (S-NC), NC12 (S-NC12), propolis aqueous extract (S-P1 or S-P2), or lignin (S-L).

Formulation	Total Phenolics*	DPPH (%)	ABTS*
Control	1.58 ± 0.4^{e}	Traces	Traces
S-NC	21.89 ± 1.0^{a}	91.02 ± 0.1^{a}	24.37 ± 0.7^{c}
S-NC12	18.53 ± 1.0^{b}	58.51 ± 1.9^{d}	44.68 ± 0.3^{a}
S-P1	$2.10 \pm 0.1^{d,e}$	66.23 ± 3.1^{c}	16.64 ± 0.2^{d}
S-P2	4.88 ± 0.4^{c}	82.27 ± 0.3^{b}	28.44 ± 0.3^{b}
S-L	2.78 ± 0.2^{d}	54.57 ± 1.2^{e}	5.48 ± 0.4^{e}

a, b, c, d: Mean with different lowercase letter in the same column indicates significant difference between films as revealed by Duncan's test, $p < 0.05$

*Total phenolics are expressed as mg of GAE/g of film.

*ABTS is expressed as µM Trolox/g of film.

3.8 Film antimicrobial activity

Figure 6 illustrates the antimicrobial activity assays. The growth inhibition zones around the S-NC, S-NC12, S-P1, S-P2, and S-L films (inhibition halos) and growth inhibition under/over the films (area of contact with the agar surface) was visually examined. Although various studies have demonstrated that propolis (Campos et al., 2014; Osés et al., 2016; Righi, Negri & Salatino, 2013), lignin (Hossain et al., 2015; Lou et al., 2012; Yang et al., 2018), and nopal cladodes (Allegra et al., 2017; Blando et al., 2019; El-Mostafa et al., 2014) exhibit antimicrobial activity against fungi and bacteria, their addition to the cassava starch film did not elicit inhibition zones of growth around the film discs for the four microorganisms tested in this study. Figure 6 also shows that bacterial colonies did not grow over the S-P1, S-P2 and S-L films, whereas all the tested microorganisms grew over the S-NC and S-NC12 films. The carbohydrates that are present at higher concentration in the S-NC and S-NC12 films could account for bacterial and yeast growth in these films. Thus, indicated a disadvantage when compare NC or NC12 to propolis extract and lignin adiition. The results observed for the S-P1, S-P2, and S-L films showed an antimicrobial action for these films (Righi, Negri & Salatino, 2013). Therefore, the use of NC in antiomcrobial packaging systems should be associated to the incorporation of antimicrobial compounds.

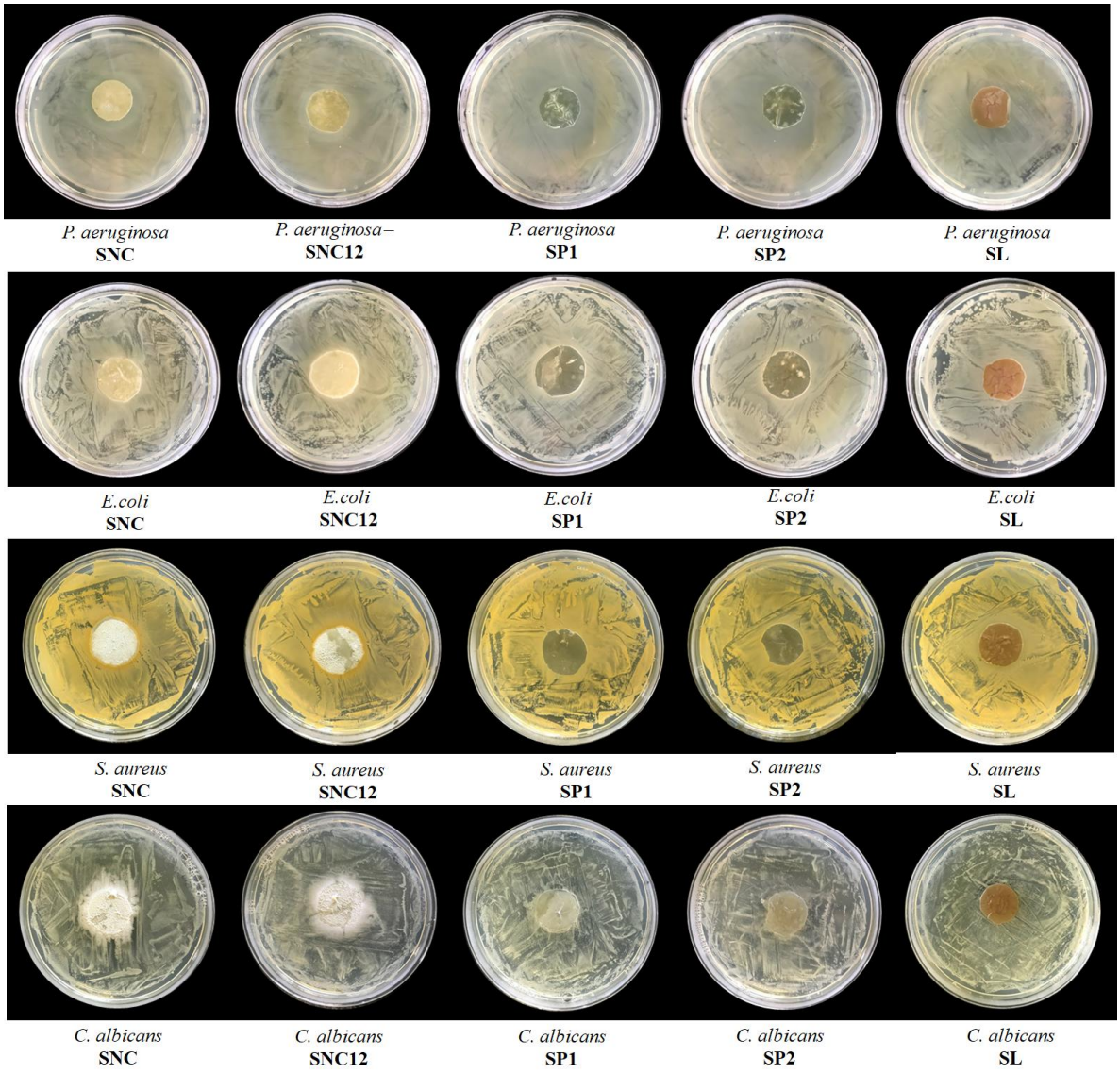

Figure 6. Antimicrobial activity assay by the agar diffusion method of the pure cassava starch film (control) and the cassava starch-based films added with NC (S-NC), NC12 (S-NC12), aqueous propolis extract (S-P1 or S-P2), and lignin (S-L) against *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans.*

4. Conclusions

Untreated nopal cladode flour (NC) and nopal cladode flour treated at pH 12 (NC12) have potential use to improve the antioxidant capacity and to enhance the opacity of cassava starch films as compared to the use of propolis extract or lignin. These two characteristics are required when obtaining packaging for food that is sensitive to oxidation. NC, propolis extract at high concentration (P2), and lignin addition slightly decreased the

cassava starch film mechanical strength and produced less elongable films. On the other hand, the films added with NC or NC12 as filler/active agent performed differently. NC12 was better incorporated into the cassava starch matrix, yielding films that were more hydrophobic, less permeable to water vapor, and more elongable than NC. Even though propolis extract and lignin also improved the cassava starch film activity antioxidant, this effect was lower as compared to NC12. Given that NC is a low-cost and highly available raw material, it can be an economically feasible alternative for use as filler/active agent in cassava starch film. However, its lack of antimicrobial activity is a disadvantage when compare to propolis extract and lignin. Therefore, we suggest that this characteristic be improved by adding antimicrobial compounds. Starch/nopal films can be employed to preserve products with high oxidation rate and susceptibility to light, including food, cosmetics, and pharmaceutical products.

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Paper 3 – Supplementary dada

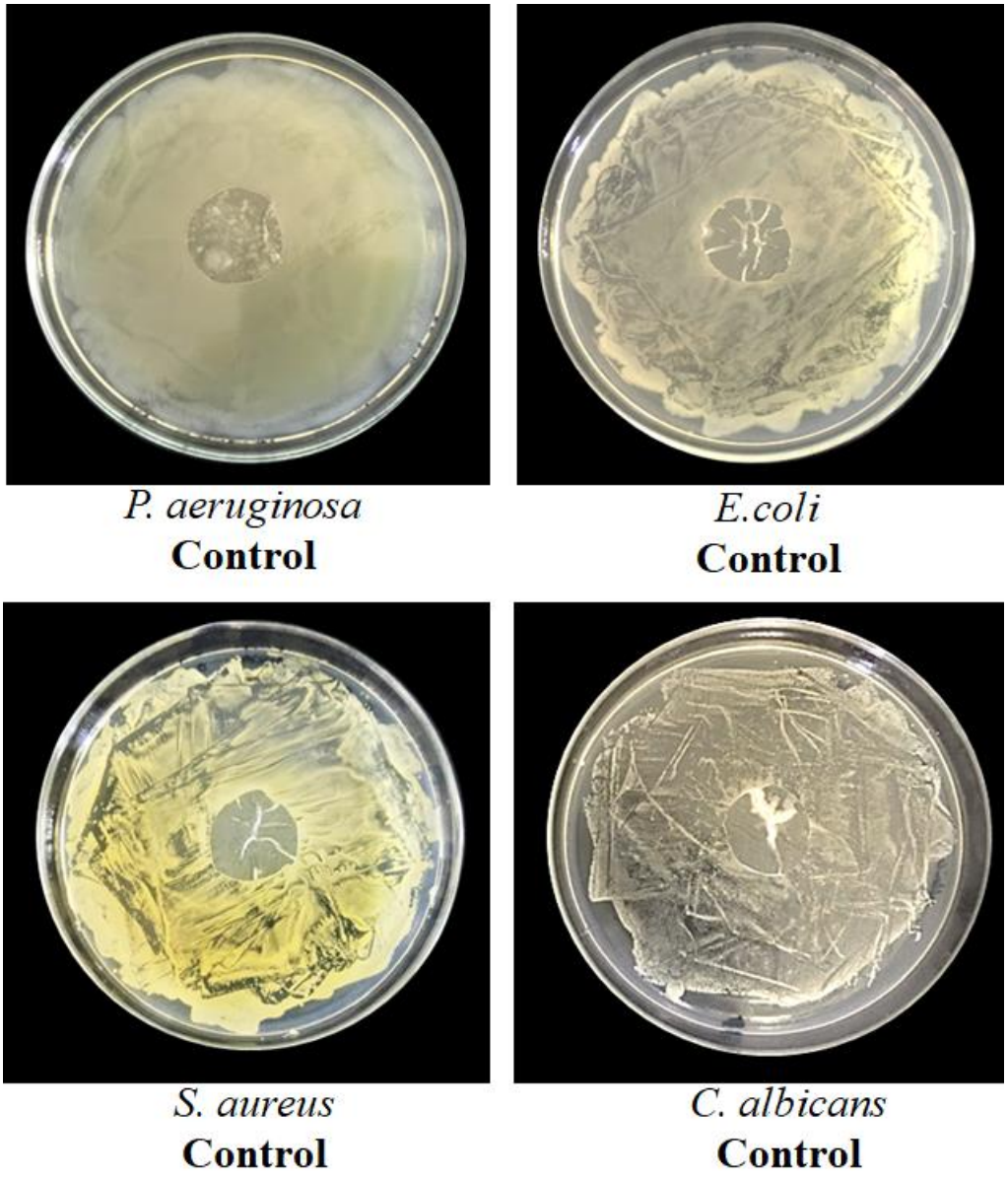

Figure 7. Antimicrobial activity assay by the agar diffusion method of the pure cassava starch film (control) against *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans.*

APPENDIX D - PAPER 4

DEVELOPMENT AND CHARACTERIZATION OF BIO-BASED FILMS INCORPORATING NATIVE CASSAVA STARCH, NOPAL FLOUR, LIGNIN AND AQUEOUS PROPOLIS EXTRACT.

Abstract

The aim of this study was to characterize and compare the bi-functional efficacy of active nopal/starch-based films produced with NC or NC12 (untreated nopal and treated under pH 12, respectively), glycerol (1.0 %), reinforced with lignin (0.4 %) and/ or activated with aqueous propolis extracts (0.6 or 1.2 %). The films were characterized using mechanical testes, moisture, opacity, solubility, surface contact angle, water vapor permeability, X-ray diffraction, active properties (total phenolic compounds, ABTS and DPPH) and antimicrobial activity against four microorganisms *S. aureus*, *E. coli*, *P. aeruginosa*, and *C. albicans.* The results achieved showed that nopal addition afforded films with yellowish color and that the alkaline treatment applied to the nopal flour (S-N12) had a greater influence in the increase of the hydrophobicity of the film surfaces and affected more the water resistance of the films than the addition of lignin and propolis extract in the tested concentrations. Despite, the films did not show inhibition zone against all of the four microorganisms tested, nopal addition contributed most with the total phenolic compounds content, and antioxidant activity by ABTS and DPPH, and afforded films with high antioxidant activity. Thus, cassava starch/nopal film is a suitable candidate for incorporation into active packaging systems reducing the oxidation process and extended the maintenance of quality of high oxidative food during storage.

Keywords: *Nopal (Opuntia ficus-indica), propolis, lignin, cassava starch-based film, antioxidant activity.*

1 Introduction

Composite materials exhibit advantages from the combination of multiple properties, which cannot be achieved by a single biopolymer and are generally obtained by adding the additives onto the continuous polymeric matrices mainly by melt blending and solution casting methods and then processed into films or sheets (Munteanu, & Vasile, 2020; Hasan, Zhao & Jiang, 2019; Jacob et al., 2020; Jacquot et al., 2014). The resultant composite material shows optimized mechanical properties (reinforcements), such as strength, stiffness, and hardness or presents antioxidant and/or antibacterial activity (Munteanu & Vasile, 2020).

Starch derived from a variety of sources (corn, potatoes, cassava and other crops) is a biopolymer inexpensive and easily accessible and has been extensively studied for the production of new bio-based materials (Bher, Auras & Schvezov, 2018; Costa, et al., 2017; Espinoza Acosta et al., 2015). Nevertheless, due its semi-crystalline nature, hydrophilicity and high solubility starch-based films are fragile, and have undesirable feature, such as poor mechanical properties, low stability when compared to synthetic materials and poor active properties, which has limited their application as food packaging of high-moisture foods and products (Colivet & Carvalho, 2017; Lim et al., 2020; Zain, Kahar & Noriman, 2016). Several studies have demonstrated that cassava-starch films can have mechanical, thermal, barrier, and bioactive properties enhanced by cross-linking reaction with reinforcement agents, such as nanocellulose or lignocellulose nanofibers (Chen, et al., 2020; Travalini et al., 2019; Zhao, Huerta, & Saldaña, 2019).

Nopal cladode flour from *Opuntia ficus-indica* is one of the most potential plants for industries to develop functional and nutritional products, creating value-added food and cosmetic (Allegra et al., 2017; González Sandoval et al., 2019; Luna-Sosa et al., 2020; Nabil et al., 2020). The inclusion of nopal flour in edible products provides an increase of calcium and fiber content and can improve the rheological properties and workability due to presence

of insoluble fibers (Cornejo-Villegas et al., 2010). These properties have suggested nopal as potential source for natural food additives such as thickening and gelling agents, acting by modifying the taste, texture, and stability of processed food (Jana et al., 2012). Nopal cladode has been also investigated as an interesting candidate for the development of biodegradable films and edible coatings (Andreu et al., 2018; Bayar, Kriaa, & Kammoun., 2016; Smida et al., 2017).

Propolis is a mixture of many components produced by honeybees with countless biological properties, such as antibacterial, antifungal, anti-inflammatory, and antioxidant activities due the large quantities of bioactive compounds, such as flavonoids and phenolic in its composition (Nina et al., 2016; Osés et al., 2016; Oliveira et al., 2015; Suleman et al., 2015). The addition of propolis extract as an antimicrobial agent in biodegradable films has been extensively studied, due to its proper incorporation into polymeric matrices and effective capacity against some microorganisms (Costa et al., 2014; Khoshnevisan et al., 2019; Suriyatem et al., 2018; Shavisi et al., 2017; Pastor et al., 2010). The addition of propolis nanoparticles also contributes to the reduction of hydrophilicity of starch-based films (Villalobos et al., 2017). According to Pérez-Vergara et al, (2020), propolis extract incorporation in cassava starch film increased opacity, producing yellowish and antifungal activity films against *Aspergillus niger*.

On the other hand, Lignin is the second most abundant natural polymer after cellulose, but it is considered the most abundant aromatic biopolymer in nature, it is also considered a polyphenolic compound, with antioxidant activity (Margida, Lashermes & Moorheada 2020; Shin et al., 2019). Several studies have been carried out regarding the incorporation of lignin as reinforcer/fillers in numerous matrices for the manufacture of polymeric products with microbial growth control and with antioxidant capacity, providing added value in the different fields (Spiridon et al., 2020; Spiridon, Anghel & Bele, 2015;

Yang et al., 2018; Zhao et al., 2019). Lignin substantially enhanced water resistance of cassava films (Narkchamnan & Sakdaronnarong, 2013). Souza de Miranda et al., (2015), reported that lignin incorporation in cassava films improved thermal, mechanical and modified structural properties, though made the material's surface rougher. Hence, the films properties are directly related to the degree of lignin association with starch.

Hence, to overcome the hydrophilicity and fragility of starch-based films new methodologies are being studied. Nevertheless, starch-based films added with nopal, lignin and propolis has not been studied up to date. Therefore, the aim of this study is to produce starch-nopal flour composite films and to evaluate the effect of propolis and lignin addition on the mechanical, functional and antioxidant activity using a friendly process without chemical residues. Furthermore, the antimicrobial activity of the composite films was also evaluated. Thus, from the outcomes we aim to show off the possibility to develop green materials from renewable resources in a sustainable way.

2. Methodology

2.1 Materials

Cassava starch (with 18 wt.% amylose and 82 wt.% amylopectin and 1×10^7 g/mol molecular weight) was purchased at the common market located in Ribeirão Preto (São Paulo, Brazil).

Glycerol and the radical scavengers DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) and ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) were supplied by Sigma-Aldrich (São Paulo, Brazil). Sodium hydroxide P.A was acquired from Dinâmica – Química Contemporânea (São Paulo, Brazil).

2.2 Nopal cladode flour production and chemical treatment

The nopal cladodes (*Opuntia ficus-indica)* were freshly harvested in the Northeastern region of Brazil (3.7327° S, 38.5270° W) at a plantation belonging to the

Animal Science Department of the Federal University of Ceará (Ceará, Brazil). From these harvested cladodes, spineless cladodes were cut into cubes, dried in an oven with forced air circulation (60 °C/12 h) (Quimis, Q314M292, Brazil), and grounded and sieved through 120-mesh sieve, to obtain the nopal cladode flour.

The nopal cladode flour (NC) was pretreated before it was used to prepare cassava starch-based films. To this end, two NC dispersions in distilled water (5 % w/v) were prepared (native pH and pH 12). Both NC and NC12 dispersions were maintained at room temperature (~25 °C) and kept in dark containers for 12 h, followed by filtration. The filtered solids were separated, and the supernatant of NC was used to prepare the composite films.

2.3 Propolis extract

The green aqueous propolis extract from *Baccharis dracunculifolia* (Muzambinho-MG, Brazil) was furnished by Laboratory of Agroindustrial Biopolymers (LBPA) located at the Chemistry Department of Faculdade de Filosofia, Ciências e Letras in Ribeirão Preto (São Paulo, Brazil). The propolis extract contained 11% dry extract, 19.8±1.4 mg/mL total phenolic compounds (expressed as gallic acid), and 4.3±0.3 mg/mL flavonoids (expressed as quercetin).

2.4 Lignin production

Lignin was extracted from sugarcane bagasse by the organosolv method with slightly acidic ethanol, as described by Jääskeläinen et al. (2017). In short, the bagasse was mixed with the extraction solution at a 10:1 (w/w) ratio in a 2-L Parr reactor (serie 4530, USA) and heated at 110 °C. After reaction for 1 h, the liquid and solid phases were separated by vacuum filtration, and lignin was precipitated with water, centrifuged, and freeze-dried for 12 h in an Alpha LSC Basic 1-4 (Christ, Germany).

2.5 Film production

Cassava starch-based films added with NC, NC12, lignin, or propolis extract were prepared. Briefly, a cassava starch filmogenic solution (4 % w/v) was obtained; glycerol (1 %) was added for all films. Next, NC and NC12 supernatant was added to prepare the S-NC and S-NC12 films. Whereas lignin 0.4 % (w/v) and propolis extract 0,6 % or 1,2 % (w/v) were added to prepare the other formulations. The lignin and propolis concentrations were established from preliminary tests (data not shown). All the suspensions were heated at 85 ± 1 °C until a semi-viscous solution emerged. The dispersions were individually cast onto an acrylic plate (0.155 g per cm^2) and dried in an oven (BOD, Marconi) at 32 °C for 10 h. The dried films were conditioned in a desiccator at 25 °C and 53% relative humidity for 48 h before they were analyzed.

2.6 Film thickness and mechanical properties

Film thickness was measured with a Zaas-Precision flat tip digital micrometer (1-μm resolution).

The mechanical tests were conducted with a texture analyzer TA TX Plus (TA Instrument, England). Tensile strength (TS) and elongation at break (E) were obtained according to the ASTM Method D882-12 (2012) by taking an average of ten determinations in each case. The films were cut into 2.54-cm-wide strips measuring at least 10 cm. The initial grip separation was 80 mm, and the crosshead speed was 1.0 mm s-1. Young's modulus (MY) was calculated as the inclination of the initial linear portion of the stress versus strain curve by using the software Texture Expert V.1.22 (SMS). All the measurements were performed in triplicate.

2.7 Film opacity

Film opacity was determined by measuring the absorbance at 600 nm on a spectrophotometer (HP Hewlett Packard 8453, USA). Each film was cut into a rectangular

piece and directly placed into the spectrophotometer test cell. An empty test cell was used as reference. Opacity was calculated by the following equation (Liu et al., 2017):

$$\text{Opacity} = A/x \quad , \qquad (1)$$

where A = absorbance at 600 nm and x = film thickness (mm). Measurements were performed in triplicate.

2.8 Film functional properties

Film solubility in water, moisture content, contact angle, and water vapor permeability (WVP) were analyzed in triplicate.

Film solubility in water was calculated as the percentage of dry matter of the solubilized films (discs with diameter of 20 mm) after immersion in 50 mL of water at 25 ± 2 °C for 24 h (Gontard, Guilbert & Cuq, 1992). The film moisture content was determined by the ASTM D644-07 Standard method (ASTM, 2007). The film contact angle was measured according to the ASTM Method D7334–08 (ASTM, 2013); OCA-20 Dataphysics (OCA 20, Dataphysics, Germany) was used. Water drop (surface tension = 72.7 mN/m) images were taken at room temperature and in air every 2 min. The image processing software GIMP 2.6.8 was used to measure the contact angle of the surface exposed to drying, which was identified as the tangent to the drop edge in the intersection of the liquid, solid, and gaseous media.

The film WVP was gravimetrically measured according to the ASTM Method E96/E96M (ASTM, 2014) at 25 ± 2 °C. The film samples were sealed over the circular opening of a permeation cell containing silica gel, and the cells were placed in desiccators containing distilled water (steady-state condition). The cell was weighed every 1 h, for 9 h, on an analytical scale. The film WVP was calculated as $WVP = w.x/t.A.\Delta P$, where "x" is the average film thickness, "A" is the film permeation area (0.00196 m^2), and "ΔP" is the difference between the partial pressure of the atmosphere over silica gel and over pure water

(3.168 kPa, at 25 °C); the term w/t was calculated by linear regression on the basis of the weight gain data as a function of time.

2.9 X-ray diffraction analysis

X-ray diffraction analyses of the films were performed on a Siemens X-ray diffractometer (Siemens, model D5005, Baden-Württemberg, Germany) operating at 40 kV and 30 mA with Kα copper radiation and monochromatic filter at room temperature. The data were collected from 2 to 50 °C on a 2θ scale at a rate of 0.02 / s. The crystallinity index (%) of the materials was quantitatively estimated as the ratio of the crystalline area of the individual peaks by the total area of the total diffractogram area; the Nara & Komiya (1983) method was followed, and the software Origin 8.1 was used (OriginPro Corporation, Massachusetts, USA).

2.10 Total phenolic compounds quantification and antioxidant activity

To determine the total phenolic compounds content and the antioxidant activity by DPPH and ABTS, deionized water was use as solvent to prepare the extract. Total phenolic compounds were determined according to Larrauri et al. (1997), with some modifications.

The antioxidant activity was determined by the DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) (Martins et al., 2012) and the ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) methods (Rufino et al., 2007). All the analyses were performed in triplicate.

2.11 Film antimicrobial activity

Film antimicrobial activity was evaluated by the agar diffusion assay according to Pelissari et al. (2009) and Shapi'i et al. (2020), with some modifications; four microorganisms were employed: *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans.* Film discs with diameter of ~1.5 cm were sterilized under UV light for 8

min on each side before the antimicrobial test was conducted. Each film disc was aseptically placed on the surface of the previously inoculated Tryptic Soy Agar (TSA) culture medium with 100 µL of suspension (MacFarland scale 1) of each microorganism. The plates were incubated at 37 °C for 24 h. The test was carried out in duplicate for each sample, on two separate occasions.

2.12 Univariate statistical analysis

From the collected data on film properties an analysis of variance (ANOVA) was conducted at a 95% confidence interval; the software Statistica 7.0 (Statsoft$^{@}$) was employed. Tukey's test was performed to evaluate average differences ($p < 0.05$) of properties between the films produced in this work.

3 Results and discussion

3.1 Film visual aspect and opacity

Figure 1 presents the visual aspect and opacity values of the cassava starch-based films produced in this work. In general, all films prepared adding nopal, propolis and lignin were visually homogeneous and easily manipulated, showing a positive interaction of all tested compounds with the cassava starch matrix. The influence of the compounds added is visually easy to observe, nopal treated (N12) gives an intense yellowish color when compared with untreated nopal (N). Pérez-Vergara et al, (2020) said that propolis extract significantly affects the color difference of cassava starch films. However, the incorporation of propolis extract (P1 and P2) in this work did not significantly affect the color, but this probably happened because the addition of nopal (N and N12) overcame the effect of adding propolis extract on the color of the films. In another hand, the addition of lignin (L) yielded darkest film with brownish shade, which agree with Michelin et al. (2020) that also reported a brownish color for lignin films added with CMC (carboxymethyl cellulose).

As already expected the addition of colored components also affect the opacity of the films. Except for the S-N12-P2 (10,82 ± 0,3 A_{600}/mm), that presented an irregular behavior when compared with the other produced films, films containing only N (13,6 ± 1,0 A_{600}/mm), N12 (12,6 ± 0,9 A_{600}/mm) and the lowest propolis extract concentration, P1 (S-N-P1: 13,9 ± 0,6 and S-N12-P1: 11,2 ± 1,4 A_{600}/mm) were the least opaque, whereas the addition of P2 and L into the cassava starch matrix significantly increased films opacity ($p < 0.05$), being the S-N-L-P2 and S-N12-L-P2 the most opaque films with 18,4 ± 1,3 and 22,2 ± 1,0 A_{600}/mm opacity values respectively.

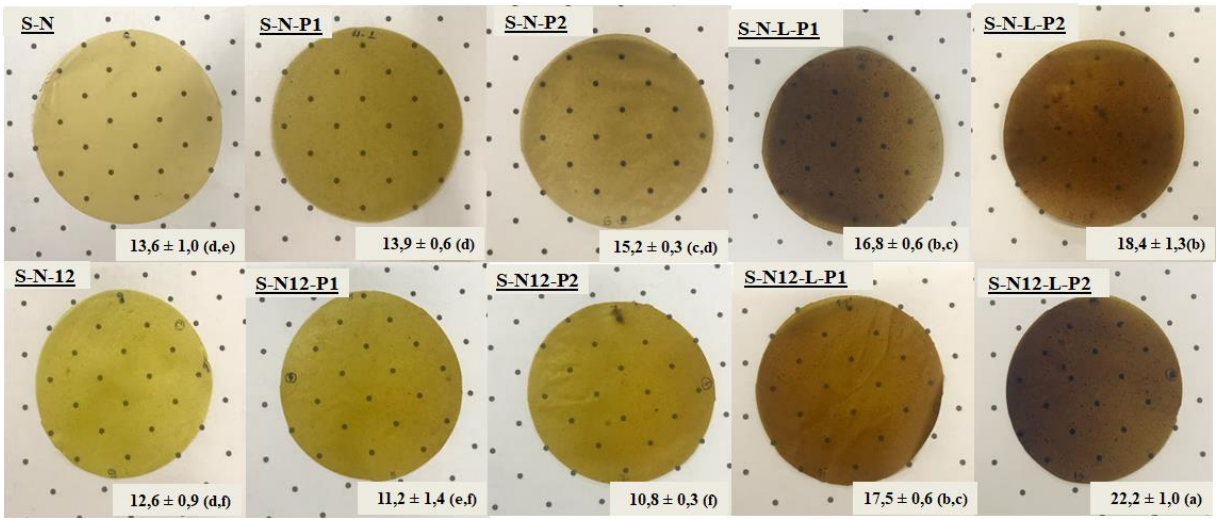

Figure 1. Visual aspect and opacity values of cassava starch-based films with addition of untreated nopal cladode flour (N), treated nopal cladode flour (N12), lignin (L) and aqueous propolis extract (P1 or P2). Opacity is expressed in A_{600}/mm. The different letters in parenthesis indicate a statistically significant difference according to Tukey's test ($p < 0.05$).

3.2 Thickness and mechanical properties.

The thicknesses of the developed films (Table 1) ranged from 0.079 ± 0.09 mm (S-N12) to 0.100 ± 0.04 mm (S-N12-P2). However, with exception of S-N12 film (0.079 ± 0.07 mm), the thickness of the films did not differ significantly ($p < 0.05$). Thus, the incorporation of propolis extract and lignin did not affect the thickness of the films.

Mechanical tests provide significant information about the stiffness or brittleness properties of the plastic films. In Table 1 it can be seen that the crosslinking among N12, L and P1 or P2 afforded an increase in tensile strength (TS) and Young's modulus (YM) of the films. Whereas, S-N12 was the most elongated film (24.23 ± 6.55 %). This result is in agreement of the obtained by González Sandoval et al., (2019) for nopal mucilage-based films. According to them, the chemical treatment applied to the nopal increases the presence of sugars that act as plasticizer, which explains a greater elongation of the film containing N12.

Nevertheless, it can be noticed that when alone, the S-N12 did not have a good mechanical behavior, however the interaction with propolis extract (P2) and lignin (L) improved the stiffness and elongation of the material. Villalobos et al., (2017) reported that low concentration of propolis nanoparticles (< 1 wt %) had a positive effect on the mechanical performance of starch-based films, including Young's modulus, tensile strength and elongation. According to them yet, at higher concentration of propolis caused a decrease in the EB but not in the Young's modulus, the same behavior it was observed in this study.

In another way, the addition of lignin on the formulations promoted a decrease on the elongation of the films, theses films showed the lowest EB values ($p < 0.05$). Therefore, it can be inferred that in general, the films loaded without lignin were better than those added of lignin in its composition. Zhang et al., (2020) achieved the same results when reinforced starch bio-composite films with lignin. Among all formulations developed, the films S-N12-L-P1 (TS: 4.76 ± 0.71 MPa, EB: 11.06 ± 2.65%, MY: 140.40 ± 0.38 MPa) and S-N12-P2 (TS: 4.36 ± 0.46 MPa, EB: 20.03 ± 2.32 %, MY: 120.63 ± 0.32 MPa) presented the best mechanical performance.

Table 1. Thickness, tensile strength (TS), elongation at break (EB), and Young´s modulus (YM) of cassava starch-based films with addition of untreated nopal cladode flour (N), treated nopal cladode flour (N12), lignin (L) and aqueous propolis extract (P1 or P2).

Film	Thickness (mm)	TS (MPa)	EB (%)	MY (MPa)
S-N	0.087 ± 0.09^{a}	3.18 ± 0.20^{d}	18.98 ± 2.16^{a}	$78.35 \pm 0.05^{c,d}$
S-N-P1	0.088 ± 0.00^{a}	$3.59 \pm 0.61^{c,d}$	$15.75 \pm 1.20^{b,c,d}$	$93.88 \pm 0.15^{b,c}$
S-N-P2	0.090 ± 0.03^{a}	3.13 ± 0.37^{d}	$18.05 \pm 1.49^{a,c}$	$72,95 \pm 0.14^{d}$
S-N-L-P1	0.096 ± 0.03^{a}	2.41 ± 0.32^{e}	$15.90 \pm 1.90^{b,c,d}$	$86.50 \pm 0.04^{c,d}$
S-N-L-P2	0.093 ± 0.08^{a}	3.31 ± 0.56^{d}	$15.76 \pm 0.70^{b,c,d}$	$101.75 \pm 0.14^{a,b,c}$
S-N12	0.079 ± 0.07^{b}	2.47 ± 0.48^{e}	24.23 ± 6.55^{a}	73.43 ± 0.33^{d}
S-N12-P1	0.089 ± 0.05^{a}	$4.12 \pm 0.91^{b,c}$	$18.61 \pm 2.37^{a,b}$	$90.98 \pm 0.36^{b,d}$
S-N12-P2	0.100 ± 0.04^{a}	$4.36 \pm 0.46^{a,b}$	$20.03 \pm 2.32^{a,b}$	120.63 ± 0.32^{a}
S-N12-L-P1	0.095 ± 0.03^{a}	4.76 ± 0.71^{a}	11.06 ± 2.65^{d}	$140.40 \pm 0.38^{a,b}$
S-N12-L-P2	0.093 ± 0.04^{a}	3.41 ± 0.37^{d}	$18.54 \pm 2.72^{a,b}$	$86.88 \pm 0.13^{c,d}$

Values with different lowercase letters in the same column are significantly different ($p < 0.05$).

3.3 Moisture, water solubility, and water vapor permeability (WVP).

The moisture content, water solubility and water vapor permeability of the composite films are shown in Table 2. Combining starch with hydrophobic compounds usually increase the water resistance of the composite biopolymer. Then, since nopal has a hydrophilic behavior, lignin and propolis are great candidates for blending with starch and nopal matrices because of its high miscibility and high degree of hydrophobicity (Ago, Ferrer, & Rojas, 2016). The moisture content of the composite films ranged from 10.41 ± 0.34 % (S-N-L-P2) to 15.08 ± 1.26 % (S-N12). Whereas the solubility showed values of 29.74 ± 4.34 (S-N) and 44.60 ± 2.05 (S-N12), it is observed that treated nopal cladode flour (N12) contributes to the highest moisture and solubility of the composite films when compared with the untreated nopal (N). The addition of lignin and propolis even reducing theses parameters, the values achieved were not significantly different according to Tukey's test ($p < 0.05$).

The WVP brings up information about the water vapor exchange between inside and outside of the film environment around the product, making possible to evaluate if the film have potential to be applied as food packaging material or as surface coating (Tavares et al., 2019). In a different way as observed for moisture and solubility, the WVP of the films

containing N12 ($0.76 \pm 0.01\ 10^{-10}$ $g.m^{-1}.s^{-1}Pa^{-1}$) was significantly lower, comparing with untreated nopal N ($1.03 \pm 0.01\ 10^{-10}$ $g.m^{-1}.s^{-1}Pa^{-1}$), for example. The reduction in water vapor permeability means a decrease in diffusion coefficient imposed by film structure, which may have a more tortuous pathway for the water vapor molecules to cross between the films surfaces (Jiang et al., 2016; Kaushik, Singh, & Verma, 2010). Thus, it can be said from the achieved results that the alkaline treatment of nopal flour affected more the water resistance of the films than the addition of lignin and propolis extract in the tested concentrations.

Table 2. Moisture content, solubility, water vapor permeability (WVP) of cassava starch-based films with addition of untreated nopal cladode flour (N), treated nopal cladode flour (N12), lignin (L) and aqueous propolis extracts (P1 or P2).

Film	Moisture (%)	Solubility (%)	WVP*
S-N	$11.76 \pm 0.25^{b,c}$	$43.62 \pm 1.69^{a,b}$	$1.03 \pm 0.01^{c,d}$
S-N-P1	$11.45 \pm 0.10^{b,c}$	29.74 ± 4.34^{c}	$0.82 \pm 0.01^{e,f}$
S-N-P2	$11.52 \pm 0.38^{b,c}$	$39.95 \pm 0.75^{a,b}$	$1.30 \pm 0.01^{a,b}$
S-N-L-P1	$11.58 \pm 0.08^{b,c}$	38.75 ± 1.31^{b}	1.37 ± 0.03^{a}
S-N-L-P2	10.41 ± 0.34^{c}	38.90 ± 0.95^{b}	$1.13 \pm 0.03^{b,c}$
S-N12	15.08 ± 1.26^{a}	$44.60 \pm 2.05^{a,b}$	$0.76 \pm 0.01^{f,g}$
S-N12-P1	$12.87 \pm 2.10^{a,c}$	46.62 ± 2.02^{a}	$0.87 \pm 0.03^{d,e,f}$
S-N12-P2	$13.70 \pm 1.04^{a,b}$	$45.06 \pm 4.91^{a,b}$	$1.03 \pm 0.03^{c,d}$
S-N12-L-P1	$14.03 \pm 0.53^{a,b}$	$43.76 \pm 3.45^{a,b}$	$0.96 \pm 0.03^{c,e}$
S-N12-L-P2	$13.95 \pm 1.62^{a,b}$	$42.77 \pm 0.20^{a,b}$	$0.95 \pm 0.02^{c,e,f}$

Values with different lowercase letters in the same column are significantly different ($p < 0.05$).
*WVP: Water Vapor Permeability ($*10^{-10}$ $g.m^{-1}.s^{-1}Pa^{-1}$).

3.4 Water contact angle (WCA)

The water contact angle degree (WCA) of the developed films surfaces are shown in Figure 2. Native starch contains hydroxyl groups and glucopyranose rings that are responsible for hydrophilic properties, thus the water contact angle of starch-based films is normally between 40 and 60 °, being classified as hydrophilic materials (Romero-Bastida et al., 2016). The WCA of the films developed in this study ranged from 26,94 ± 0,56 ° (S-N) to 74,18 ± 0,07 ° (S-N12-L-P2). Luna-Sosa et al., (2020) also reported a small WCA (37.12 ± 9.92 °) when mucilage with fibers of untreated nopal was added in pectin-based films.

It is very clear the huge difference in the WCA formed on the surfaces of the S-N (26, 94 ± 0,56 °) and S-N12 (70,85 ± 0,11 °) films, which indicates that alkaline treatment on the nopal cladodes flour was significantly to the increase of the hydrophobicity of the film surfaces. Despite the addition of propolis extract tends to change the films surface properties to more hydrophobic domains, effect associated with the presence of wax and resin compounds (Villalobos et al., 2017), this it was only observed for the S-N based-films, when propolis extract it was added in higher concentration (S-N-P2: 47,63 ± 0,69 °). Lignin had s small contribution in the increase of hydrophobicity of the S-N based films surfaces.

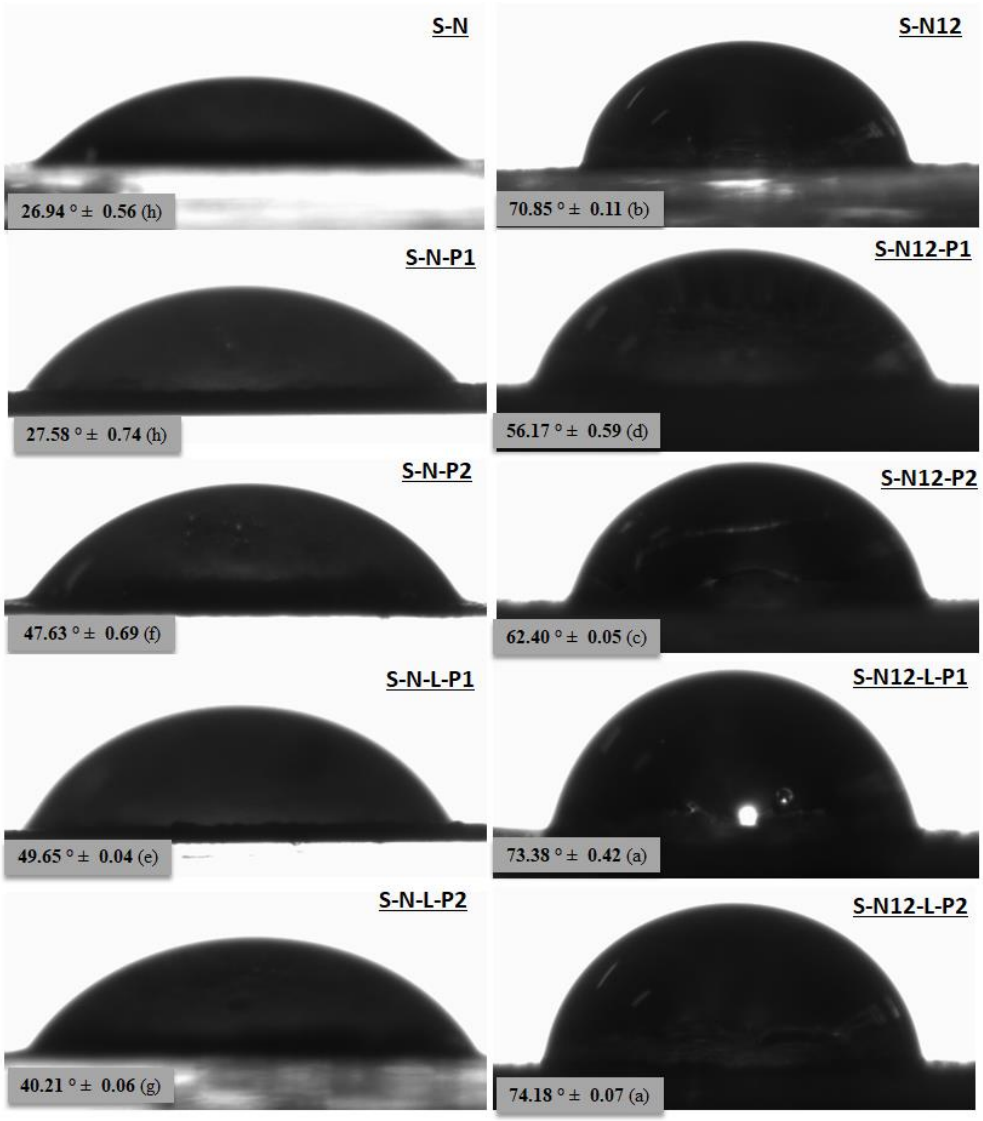

Figure 2. Water drop deposited on the film surface the contact angle degree (θ) of cassava starch-based films with addition of untreated nopal cladode flour (N), treated nopal cladode flour (N12), lignin (L) and aqueous propolis extract (P1 or P2). The different letters in parenthesis indicate a statistically significant difference according to Tukey's test ($p < 0.05$).

3.5 X-ray diffraction (XRD)

The wide-angle X-ray diffraction patterns of cassava starch/nopal-based films reinforced with propolis extract and are shown in Figure 3. The S-N and S-N12 films exhibited diffraction peaks at 2θ = 15º, 20º, 25°, 30º, and 38º. These peaks are similar to those

found by Travalini et al., (2019), who studied cassava starch-based films. However in S-N12 film the peaks presented less intensity (Fig. 3, A), which is maybe related to a decrease in crystallinity after the alkaline treatment (S-N: 23,2 % and S-N12: 19,4 %). Starch-based films present a broad peak around 20°, indicating an amorphous zone due to the semi-crystalline state of starch granules (Quin et al., 2019).

Due the cross-linked between glycerol and amylose, starch-based materials form crystals known as type V, and have crystalline fraction between 15% and 45%, depending on the starch origin (Campos et al., 2013). The degree of the starch hydration also affects the crystallization, being classified as V_A type, less hydrated, and V_H for more hydrated starches (Campos et al., 2017). Cassava starches and other tuberculous ones, presents though, a hexagonal structure called type B (Waterschoot et al., 2015). The intensity of the peaks correlated with increased crystallinity, indicates better interaction between cassava starch (S) and untreated nopal (N) (Travalini et al., 2019). Despite the difference in intensity of the peaks, no shifts in the blends peaks are observed in the diffraction patterns of the composite films, showing a similar molecular interaction.

When evaluated the incorporation of lignin and propolis in S-N and S-N12 based films separated (Fig. 3, B and Fig. 3, C, respectively) we noticed the presence of one unknown new peak around $2\theta = 10^{o}$ only for the S-N-P1 and S-N-P2 formulations (Fig. 3, B), likewise observed by Suriyatem et al., (2018) who also added propolis extract in a starch matrix. In can be observed in Fig. 3, C, that the formulation S-N12-L-P2 (17,9 %) showed the lowest crystallinity profile. Since lignin is an amorphous polymer (Souza de Miranda et al., 2015) and S-N12 showed as an amorphous material, this reduction in crystallinity was expected.

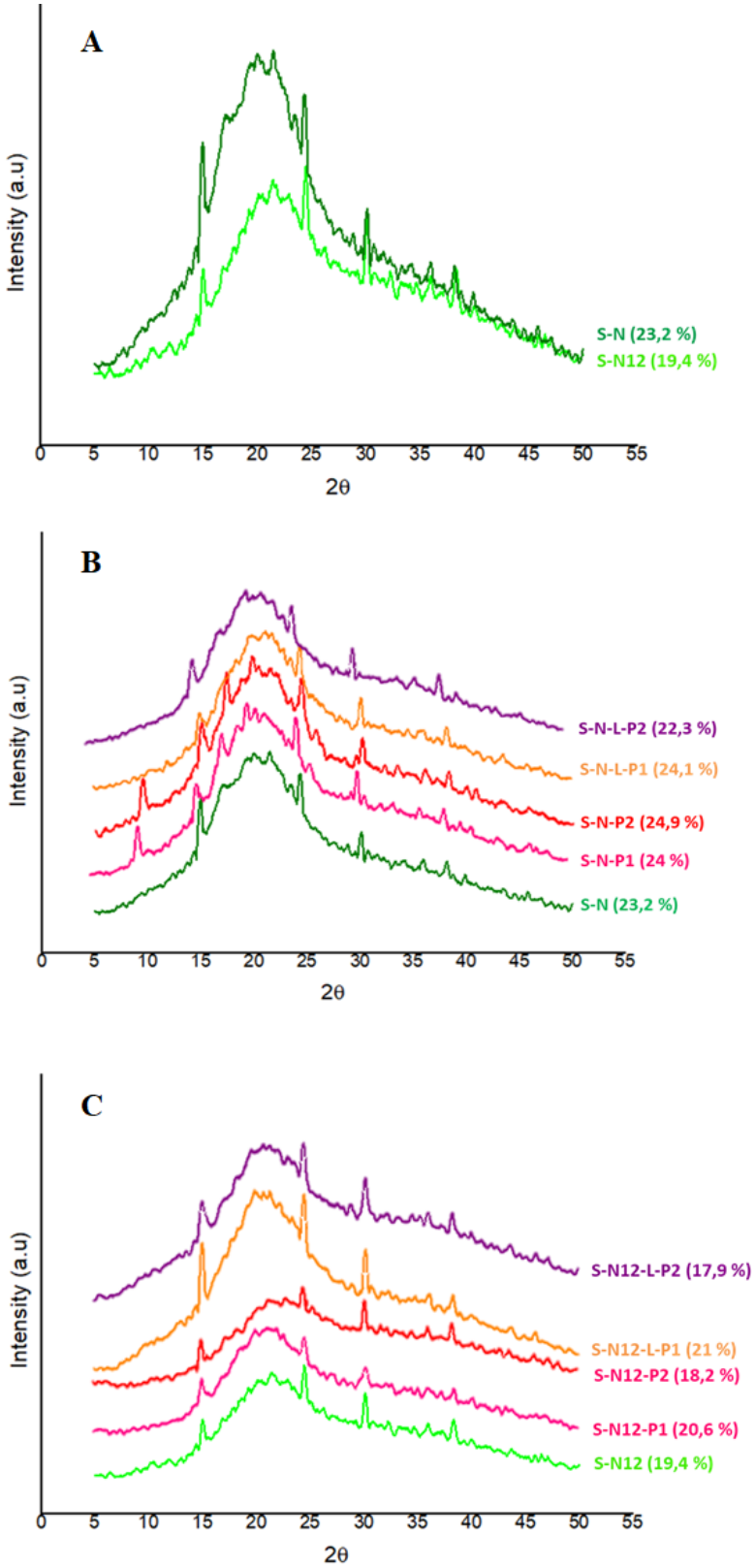

Figure 3. X-ray diffraction and percentage of crystallinity of cassava starch-based films with addition of untreated nopal cladode flour (N), treated nopal cladode flour (N12), lignin (L) and aqueous propolis extract (P1 or P2). A - Comparison between S-N and S-N12 films; B - Comparison among starch based-films added with untreated nopal cladode flour (N), and C - Comparison among starch based-films added with treated nopal cladode flour (N12).

3.6 Total phenolic compounds content and antioxidant capacity

Natural antioxidant or isolated substances with antioxidant compounds when incorporated into biopolymeric matrices can assist in the development of smart and biodegradable packaging (Assis et al., 2017).

Table 3 exhibits the bioactive function, total phenolics determination and radical scavenging activity by DPPH and $ABTS^{\bullet+}$ assays, uptake for the composite films. It can be seen by analyzing separated S-N and S-N12 films show different behaviors. Whereas, S-N based films showed higher values for total phenolics and DPPH, S-N12 displayed higher values for ABTS antioxidant activity. While S-N showed values of 21.89 ± 1.0 mg of GAE/g of film, 91.02 ± 0.1 % and 24.37 ± 0.7 µM Trolox/g of film for total Phenolics, DPPH and ABTS, respectively, S-N12 presented values of 18.53 ± 1.0 mg of GAE/g of film, 58.51 ± 1.9 % and 44.68 ± 0.3 µM Trolox/g of film for total Phenolics, DPPH and ABTS, respectively. According to Dastmalchi et al., (2011) and Maniglia & Tapia-Blácido, (2019), $ABTS^{\bullet+}$ and DPPH evaluates antioxidant compounds with different hydrophilicity degree, whilst the DPPH method can only measure compounds of hydrophilic nature, $ABTS^{\bullet+}$ is a broader method that is able to valuate lipophilic and hydrophilic antioxidant compounds.

Table 3 also makes clear that in general, the addition of propolis and lignin did not significantly increase the antioxidant capacity of the blended films with the exception of the $ABTS^{\bullet+}$ of the S-N-P2 (38.32 ± 0.3 µM Trolox/g of film) and of the total phenolic of the S-N12-L-P2 (20.13 ± 0.7 mg of GAE/g of film) that are significantly higher ($p < 0.05$). Similar results was already reported for lignin, that at low concentration did not increase the antioxidant capacity of gelatin-based and CMC-based films (Aadil, Barapatre & Jha, 2016; Michelin et al., 2020).

According Caetano et al., (2017), neat cassava starch film showed an antioxidant activity measured by DPPH scavenging activity of 13.0 ± 0.6 %. However, in this study the

pure cassava starch film did not show significantly antioxidant activity, data not shown. Thus, it could be conclude that nopal (untreated, N or after alkaline treatment, N12) contributed most with the total phenolic compounds content, and antioxidant activity by ABTS and DPPH of the developed composite films and then, can be considered a suitable candidate for incorporation into active packaging systems assisting in the maintenance of quality of high oxidative food during storage.

Table 3. Total phenolics determination and radical scavenging activity of cassava starch-based films with addition of untreated nopal cladode flour (N), treated nopal cladode flour (N12), lignin (L) and aqueous propolis extract (P1 or P2).

Formulation	Total Phenolics*	DPPH (%)	ABTS*
S-N	$21.89 \pm 1.0^{a,b}$	91.02 ± 0.1^{a}	24.37 ± 0.7^{e}
S-N-P1	$21.41 \pm 0.3^{a,e}$	90.97 ± 0.3^{a}	36.23 ± 0.3^{c}
S-N-P2	22.34 ± 0.3^{a}	90.59 ± 0.3^{a}	38.32 ± 0.3^{b}
S-N-L-P1	$20.86 \pm 1.1^{a,c}$	90.31 ± 0.3^{a}	29.37 ± 0.1^{d}
S-N-L-P2	$21.83 \pm 0.1^{a,d}$	90.48 ± 0.9^{a}	30.01 ± 0.8^{d}
S-N12	$18.53 \pm 1.0^{f,g}$	58.51 ± 1.9^{b}	44.68 ± 0.3^{a}
S-N12-P1	$19.80 \pm 1.1^{c,d,g}$	53.20 ± 1.6^{c}	44.48 ± 0.2^{a}
S-N12-P2	$19.83 \pm 0.5^{b,c,d,e,g}$	57.58 ± 1.8^{b}	44.57 ± 0.2^{a}
S-N12-L-P1	$18.79 \pm 0.2^{c,g}$	$56.49 \pm 1.4^{b,c}$	44.81 ± 0.1^{a}
S-N12-L-P2	$20.13 \pm 0.7^{b,c,d,e,f}$	$56.05 \pm 1.8^{b,c}$	44.82 ± 0.4^{a}

Values with different lowercase letters in the same column are significantly different ($p < 0.05$).
*Total phenolics are expressed as mg of GAE/g of film.
*ABTS is expressed as µM Trolox/g of film.

3.7 Film antimicrobial activity

Figure 4 displays the results achieved for the antimicrobial activity assay of the developed composite films formulations with untreated nopal (N), nopal after alkaline treatment under pH 12 (N12) and with lignin and highest concentration of propolis extract (S-N-L-P2 and S-N12-L-P2). The antimicrobial activity details of all composite films produced in our study are shown in supplementary data (Figure 5.SD A and B).

Despite recognized antimicrobial activity of propolis against fungi and/or bacteria, (Campos et al., 2014; Osés et al., 2016; Pérez-Vergara et al., 2020), lignin (Hossain et al.,

2015; Yang et al., 2018), and nopal cladodes (Allegra et al., 2017; Blando et al., 2019; El-Mostafa et al., 2014), in this study no inhibition zone were observed against all of the four microorganisms tested *S. aureus*, *E. coli*, *P.aeruginosa*, and *C. albicans.* With the exception of the *P.aeruginosa* it was observed a microbial growth over the surface of the films. Since it seems to have an optimal concentration of the antimicrobial agent depending on polymeric matrix and film composition for antibacterial effect (Correa-Pacheco et al., 2019; Pérez-Vergara et al., 2020); thus, to overcome this hurdle for cassava starch/nopal-based films, the results suggest that the incorporation of other concentrations of propolis extract, lignin and/or other compound with recognized antimicrobial activity should be evaluated.

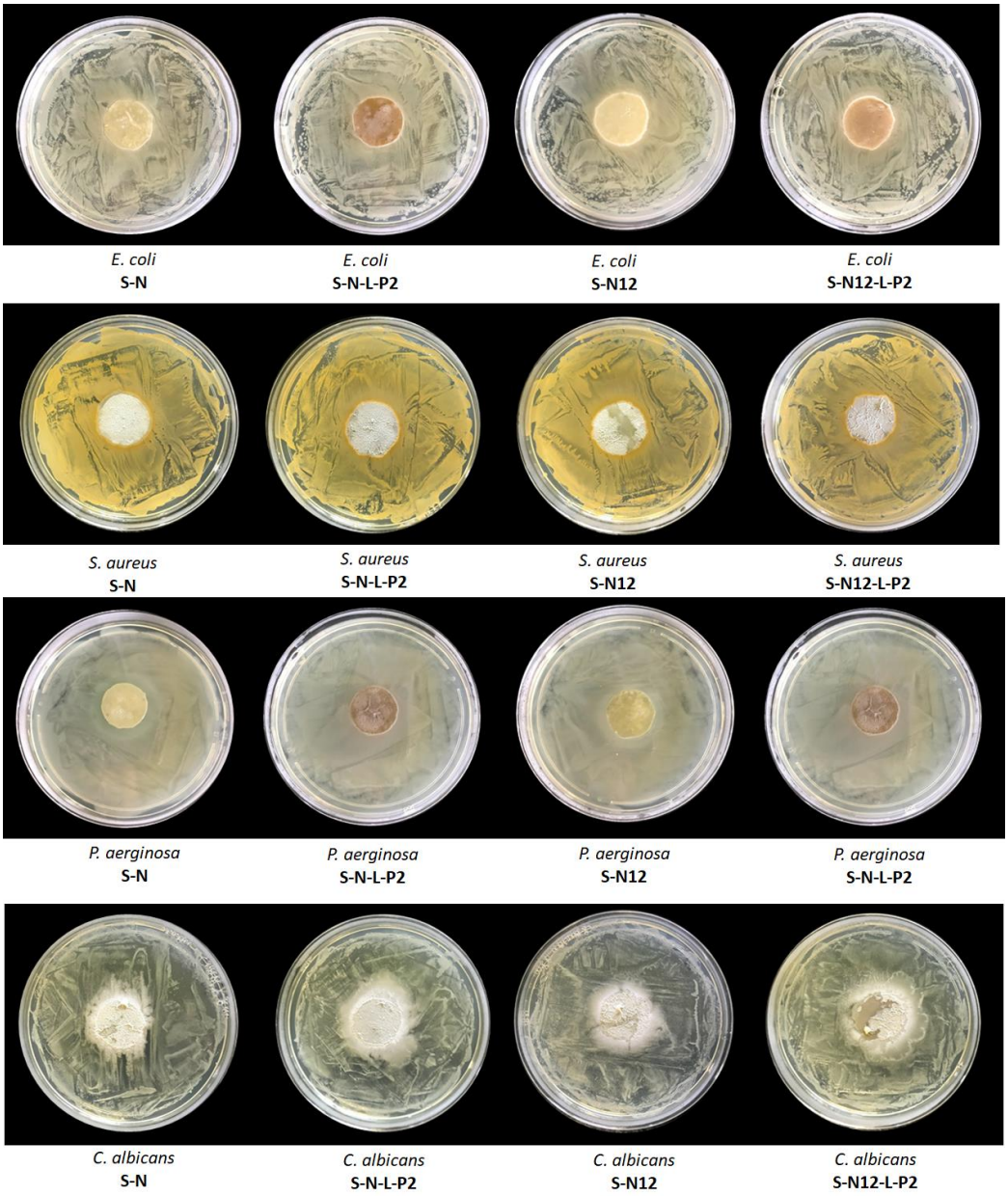

Figure 4. Antimicrobial activity assay by the agar diffusion method of cassava starch-based films with addition of untreated nopal cladode flour (N), treated nopal cladode flour (N12), lignin (L) and aqueous propolis extract (P1 or P2) against *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans.*

4 Conclusions

Alkaline treatment under pH 12 applied to the nopal flour (N12) had a greater influence in the surface hydrophilicity and in the water resistance of the films than the addition of lignin and propolis extract in the tested concentrations. Also, despite the antimicrobial activity did not showed inhibition zone against all of the four microorganisms

tested *S. aureus, E. coli, P.aeruginosa,* and *C. albicans,* this results can be overcome by testing the incorporation of higher concentration of propolis extract, lignin and/or other compound with recognized antimicrobial activity.

In addition, it could be also conclude that nopal contributed most with the total phenolic compounds content, and antioxidant activity by ABTS and DPPH, and afforded films with high antioxidant activity. S-N showed values of 21.89 ± 1.0 mg of GAE/g of film, 91.02 ± 0.1 % and 24.37 ± 0.7 µM Trolox/g of film for total Phenolics, DPPH and ABTS, respectively, and S-N12 presented values of 18.53 ± 1.0 mg of GAE/g of film, 58.51 ± 1.9 % and 44.68 ± 0.3 µM Trolox/g of film for total Phenolics, DPPH and ABTS, respectively. Thus, cassava starch/nopal film is a suitable candidate for incorporation into active packaging systems assisting in the maintenance of quality of high oxidative food during storage.

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Paper 4 – Supplementary dada

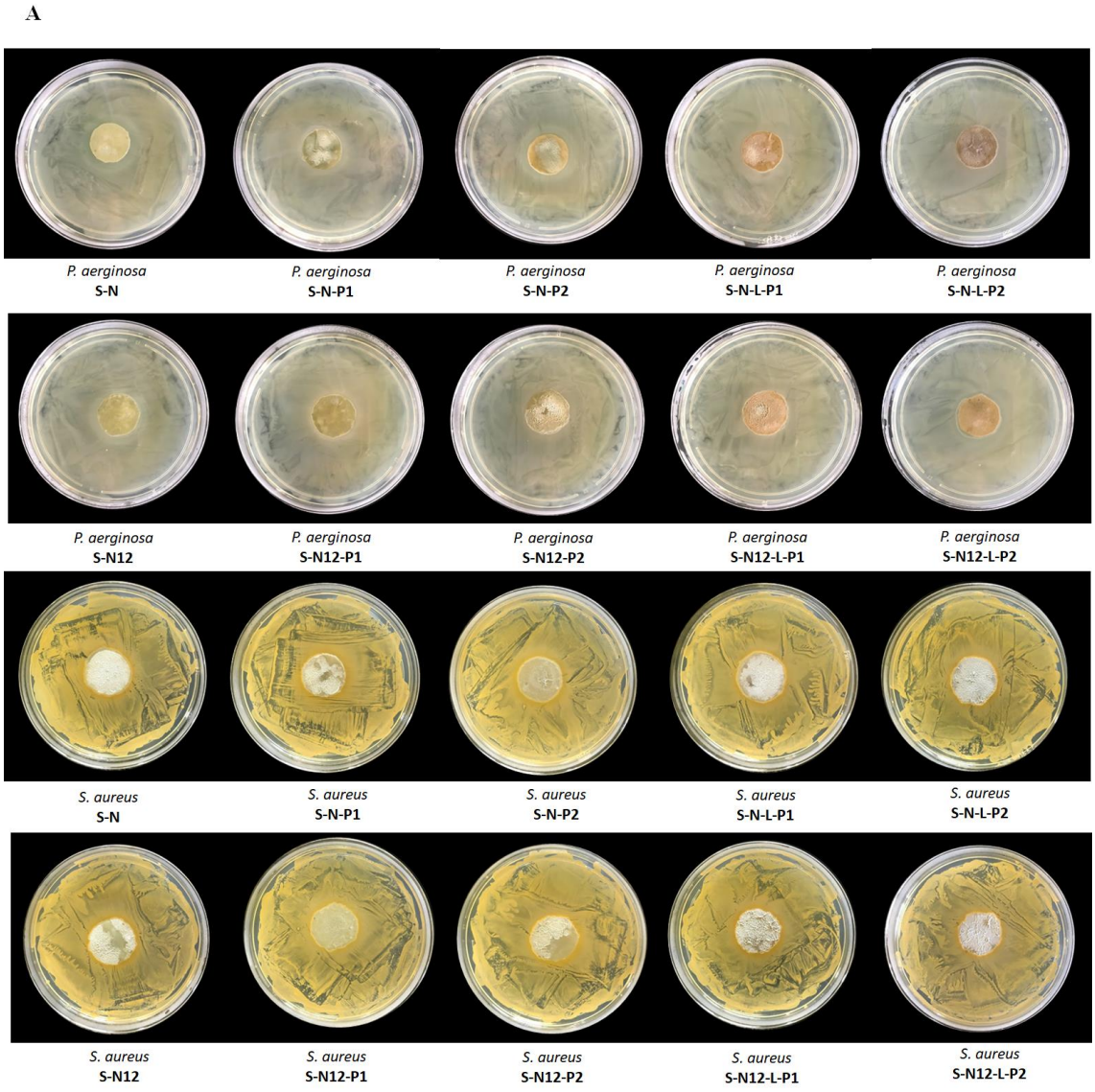

Figure 5.SD (A). Antimicrobial activity assay by the agar diffusion method of cassava starch-based films with addition of untreated nopal cladode flour (N), treated nopal cladode flour (N12), lignin (L) and aqueous propolis extract (P1 or P2) against *Pseudomonas aeruginosa* and *Staphylococcus aureus.*

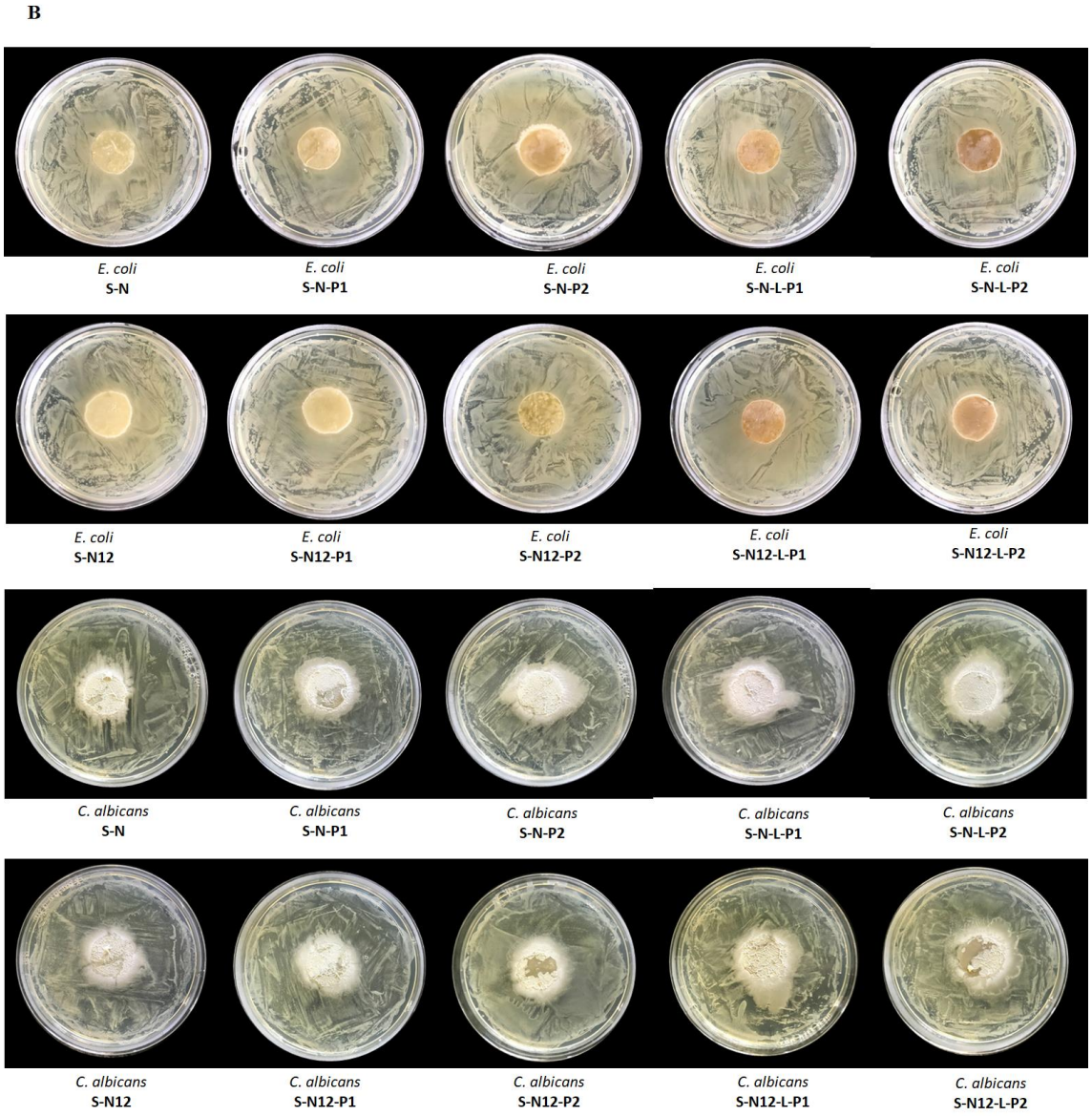

Figure 5.SD (B). Antimicrobial activity assay by the agar diffusion method of cassava starch-based films with addition of untreated nopal cladode flour (N), treated nopal cladode flour (N12), lignin (L) and aqueous propolis extract (P1 or P2) against *Escherichia coli* and *Candida albicans.*