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Phenolic Compounds from *Psidium guajava* (Linn.) Leaves: Effect of the Extraction-Assisted Method Upon Total Phenolics Content and Antioxidant Activity

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Abstract: The aim of this study was to investigate the influence of the extraction method on the dry extract yield of guava leaves, correlating the total phenolic content (TPC) with the antioxidant activity. The dry extracts were obtained from hydroethanolic (50 and 70%) extract using the ultrasound-assisted method. Folin-Ciocalteau reagent was used to determine the content of TPC. DPPH (2,2-diphenyl-1-picrylhydrazyl) in vitro assay was used to determine the ability to scavenge free radicals. The results analyses demonstrated that the ultrasound-assisted method produced a higher yield in both dry extracts (11%), in contrast to the conventional method. The 50% hydroethanolic solvent was more efficient in the extraction of bioactive compounds. Both extracts showed a positive correlation of phenolic content with antioxidant activity. The FTIR spectrograms showed changes in the chemical groups, as well as determining the aromaticity index of the extracts, indicating a higher aromatic prevalence to the solvent 50%, although it presented simpler phenolic structures. In conclusion, the results provide an important basis for the use of phenolic compounds extracted from guava leaves, not only due to the antioxidant activity exerted, however, for potential use as a crosslinking agent of sulfated and non-sulfated glycosaminoglycan (GAG).

Keywords: *Psidium guajava* Linn.; hydroethanolic solvent; ultrasound-assisted; phenolic compounds; antioxidant activity; aromaticity index.

1. Introduction

Psidium guajava (guava) is a plant that can reach 8 to 10 meters in height; it belongs to the phylum Magnoliophyta, class Magnoliopsida, and family Myrtaceae. The guava is found in many tropical and subtropical regions [1,2].

Different parts of this plant are widely used worldwide, either as food or in traditional medicine [3]. Given its use in health practices, *in vitro* and *in vivo* pharmacological research has been widely used to demonstrate the potential effect of guava leaf extracts in helping to treat a variety of highly prevalent diseases, investigating the use of extracts by traditional medicine in cases such as diabetes mellitus, cardiovascular diseases, cancer, diarrhea, bacterial infection, and antiparasitic [4,5]. The biological activities of guava extracts have been attributed to secondary metabolic compounds, mainly to phenolic compounds [6].

Phenolic compounds represent a wide variety of substances distributed in the plant kingdom and are the most abundant secondary metabolites of plants, with more than 8000 phenolic structures already detected, from simple molecules, such as phenolic acids, to highly

polymerized substances, such as tannins [7]. The chemical structure of phenolic compounds is characterized by the presence of one or more aromatic rings attached to at least one hydroxyl radical and/or other functional substitutes, and can also be divided according to the number of phenolic rings and the structures to which they are attached linked; these characteristics confer antioxidant properties to these compounds [8].

Antioxidants are defined as molecules that can slow, inhibit, or prevent the oxidation process, eliminating free radicals and decreasing oxidative stress [9]. Oxidative stress is characterized as an imbalance in which excessive amounts of reactive oxygen and/or nitrogen species (ROS/RNS), such as hydroxyl radical (· OH), superoxide anion (O^{2-}), hydrogen peroxide (H_2O_2), and peroxy-nitrite ($ONOO^-$) exceeds the endogenous capacity, resulting in the oxidation of a variety of biomacromolecules [10,11].

The antioxidant properties of phenolic compounds can be mediated by three mechanisms: (I) eliminate radical species such as ROS/RNS; (II) suppress the formation of ROS/RNS by inhibiting some enzymes or chelating trace metals involved in the production of free radicals; and (III) regulate or protect the antioxidant defense [12,10].

In addition to the antioxidant activity expressed by phenolic compounds, due to their high compatibility with free radicals, these compounds have potential use in the chemical crosslinking process due to their structure composed of hydroxyl groups and other functional groups, such as carboxyl and sulfated groups, being able to effectively perform intermolecular interactions with proteins and glycosaminoglycans [13], like collagen, hyaluronan, chondroitin sulfate, and ulvan.

Chemical crosslinking is a highly versatile method with good mechanical stability for the manufacture of hydrogels [14], films [15], and scaffolds [16]. Sodium periodate and aldehyde compounds are generally used for this purpose, such as glutaraldehyde and formaldehyde. Although they are very good crosslinking agents, they are not preferable due to their physiological toxicity [17]. Therefore, the search for biocompatible natural compounds has been indicated as an important alternative for use as crosslinking agents.

Phenolic compounds have been extracted using conventional methods, such as maceration, infusion, percolation, countercurrent extraction, and Soxhlet. However, these methods have some disadvantages, such as long extraction times, requiring relatively large amounts of plant samples, possible degradation or transformation of bioactive compounds, and higher consumption of organic solvent [18,19].

In a study carried out by Seo *et al*. (2014) [11], hydroethanolic extracts (50 and 70%) were prepared with guava leaves by the maceration method. To obtain the extract, 100 g of leaves were used in 1.5 L of solvent for 4 days at room temperature. The results of this study confirmed the contents of TPC of 185 mg GAE/g and 150 mg GAE/g for hydroethanolic extracts 50 and 70%, respectively.

In contrast to conventional methods, ultrasound-assisted extraction has been widely requested for applications in the food and pharmaceutical industries, as it presents advantages combined with greater productivity, yield, and selectivity of the compounds of interest [20].

Given the importance of the antioxidant activity exerted by the phenolic compounds present in guava leaves and their potential use as a crosslinking agent, this study aimed to investigate the effects of the ultrasound-assisted method to optimize the extraction of TPC from hydroethanolic extracts. In addition, correlate the TPC with the ability to eliminate free radicals *in vitro*. We hypothesized that TPC is capable of crosslinking glycosaminoglycans after the partial reaction between GAG and sodium periodate ($NaIO_4$).

2. Materials and Methods

2.1. Materials.

The leaves of the guava plant (*Psidium guajava* Linn. var. *Pomifera*) from the seedling bed was kindly donated by the Division of Parks and Conservation Units of the Secretariat for the Environment (Sorocaba, São Paulo, Brazil), ethanol (Synth, São Paulo, Brazil), hydrochloric acid (Neon, São Paulo, Brazil). Folin-Ciocalteau reagent (Dinâmica, São Paulo, Brazil), sodium carbonate (Dinâmica, São Paulo, Brazil), gallic acid (Dinâmica, São Paulo, Brazil). 2,2-diphenyl-1-picrylhydrazyl (Sigma-Aldrich, Steinheim, Germany), methanol (Chemco, São Paulo, Brazil). All reagents used were analytical grade.

2.2. Obtaining plant material.

The guava leaves were harvested by hand, carefully packed for transport, and on the same day, the leaves were handled to remove debris and washed to remove impurities. The leaves were rinsed with 70% ethanol to reduce drying time and kept in an oven with forced air circulation (Tecnal, TE-394, São Paulo, Brazil) at 37 °C to constant weight. The dried leaves were ground, and the powder was standardized using a standard sieve (20 mesh, 0.833 mm), and stored in an airtight container until the studies were carried out.

2.3. Extraction of total phenolic content.

The ultrasound-assisted extraction was carried out using as solvent hydroethanolic 50% and 70% v/v, acidified with hydrochloric acid (0.5% v/v), and application of ultrasonic wave single frequency (40 kHz). For the extraction, 10 g of standardized guava leaf powder (SGLP) was placed in Erlenmeyer and dispersed in 150 ml of previously acidified ethanol 50% v/v and kept in an ultrasound bath (Unique, USC-3300, São Paulo, Brazil) for 60 min., at 30 °C. The same process was carried out using ethanol 70% v/v previously acidified. Both dispersions were vacuum filtered (Whatman® cotton filter, grade 44: 3 μm) and stored away from light and heat. The residue retained in the cotton filter was reprocessed using the same extraction conditions already described. The extracts from the first and second extraction processes were mixes.

The solvent was eliminated using a rotatory evaporator (Tecnal, TE-211, São Paulo, Brazil). The concentrated extract was resuspended in ultrapure water and dried by lyophilization (Liofilizador - L101, Liotop, São Paulo, Brazil). The dry extract of guava leaves from acidified ethanol 50% (DEGL-50) e ethanol 70% (DEGL-70) were stored away from humidity, light, and heat.

The mass yield of the dry extracts was calculated according to Eq. (1).

$$Dry\ extract\ yield\ (\%) = \frac{Dry\ extracts\ (g)}{Powder\ guava\ leaves\ (g)}\ x\ 100 \quad (1)$$

2.4. Determination of total soluble phenolic compounds.

The total soluble phenolic content from DEGL-50 and DEGL-70 samples were determined using the Folin-Ciocalteau colorimetric method described by Çam and İçyer (2015) [21] with modifications. The dry extract was solubilized in ultrapure water at a concentration of 1 mg/mL. This solution was filtered through a cotton filter, discarding the first 25 mL. The

filtrate as the standard solution (SS - 0.001g/mL). After that, an aliquot of 50 µL the standard solution was collected and transferred to a test tube with 2.5 mL of the Folin-Ciocalteau reagent being added. The mixture was slightly stirred, and 2 mL of a solution of anhydrous sodium carbonate (Na_2CO_3) at 7.5% (w/v) was added. The mixture was incubated for 15 minutes in a thermostatic bath (Brookfield, TC 550, Middleborough, USA) at 50 °C and finally cooled in a freezer at -18 °C for 5 min.

The samples were analyzed in triplicate, and the TPC was determined at a wavelength of 760 nm in a UV/Vis-spectrophotometer (Femto 800XI, São Paulo, Brazil), using FemtoScan Software. For greater reliability of the assay, a white reagent (ultrapure water) was conducted under the same conditions.

For the quantification of TPC, the research group previously constructed an analytical curve with concentration points between 50 – 400 µg/mL of gallic acid, and the results were expressed in mg/g gallic acid equivalent (GAE) of the samples [22].

2.5. In vitro antioxidant activity.

The antioxidant activity of the TPC in the DEGL-50 and DEGL-70 samples were determined using a solution 50 µL of each dry extract (1 mg/mL) and mixed with 2 mL of 100 µM DPPH (4 mg/100 mL methanol) as free radical. The mixture was placed in the dark at room temperature for 60 minutes. After that, the absorbance of the samples was analyzed in triplicate at 520 nm by UV/Vis-spectrophotometer, using purified water for zero adjustments [23].

Assessment of the scavenging abilities phenolic content on DPPH expressed as percent inhibition was calculated according to Eq. (2).

$$DPPH\ scavenging\ effect\ (\%) = \frac{Abs1 - Abs2}{Abs1}\ x\ 100 \qquad (2)$$

Where Abs1 represents the absorbance of the control at 60 minutes, and Abs2 is the absorbance of the sample at 60 minutes.

2.6. Fourier transform infrared spectroscopy (FTIR).

The FTIR analysis (Shimadzu, IRAffinity-1, Kyoto, Japan) was used to collect FTIR spectra via LabSolutions Software v.2.10. The KBr discs were prepared using a hydrostatic press, with a mass of 2 mg of sample (dry extract) mixed with 300 mg of KBr. The FTIR spectra were collected between 4000 – 400 cm^{-1}, with a resolution of 4 cm^{-1} and 32 scans.

2.7. Determination of the aromaticity index by FTIR.

The aromaticity index provides an estimate of the aromatic character of the analyzed samples, through the ratio between the peak absorption intensity around 1620 cm^{-1}, attributed to the aromatic groups, with the peak absorption n intensity around 2920 cm^{-1}, which represents aliphatic groups [24,25]. The absorption intensity values were obtained using FTIR spectra and measured using Labsolutions Software v.2.10. The sample's aromaticity index was calculated according to Eq. (3).

$$Aromaticity\ index\ (AI) = \frac{I1620}{I2920} \qquad (3)$$

Where I-1620 represents the absorption intensity in the wavenumber around 1620 cm^{-1} and I-2920 is the absorption intensity in the wavenumber around 2920 cm^{-1}.

3. Results and Discussion

3.1. Efficiency of ultrasound-assisted extraction method.

For the extraction of TPC from leaves of different species of plant, the most used solvents are the water and the organic solvents such as ethanol, methanol chloroform, hexane, ethyl acetate, and acetone. Because of the diversity of available solvents, the choice of the most suitable solvent has become a crucial factor, both to increase the yield in TPC, to improve biological safety, and environmentally friendly [6].

The yield of the ultrasound-assisted extraction (UAE) method measured by the dry extract weight after freeze-drying was 11.4% for DEGL-50 and for DEGL-70 and 11.7% for DEGL-70%.

Dry extracts obtained by the maceration method in ethanol 50% [26] and ethanol 70% [27] were respectively 1.66% (three days of maceration) and 2.75% (four days of maceration). Unlike the methods of extraction by maceration where the time and dielectric constant (Ɛr) of ethanol 50% (Ɛr ~ 52) and 70% (Ɛr ~ 40), at a temperature of 25 °C, influenced the process yield, the UAE method for 60 min at 30 °C, a frequency of 40 kHz have not affected by the solvent Ɛr. The powerfulness of the UAE method compared to maceration, can be explained by the effect of ultrasonic energy through a solvent system containing solid particles. So, the energy in the form of waves generates a perpendicular or parallel force on the liquid surface, that converts the sonic energy into mechanical energy, equivalent to several thousand of atmospheric pressures, which is responsible for breaking the cell wall and dissolving phenolic compounds and another soluble in a specific solvent.

3.2. Determination of total phenolic compounds.

The analytical curve was developed to determine TPC (Figure 1). The determination coefficient, designated R^2, was observed in the concentration range of 50 – 400 µg/mL. The data from the linear regression analysis for the calibration graph showed a good coefficient of determination was 0.998, and the linear regression equation was y = 0.0017x - 0.0832 (22). In these conditions, the analytical validation method by UV/Vis-spectrophotometry to determine the polyphenol content proved that the method was linear, reliable, robust, reproducible, easy to perform, and low cost. This methodology complies with the requirements for analytical application and to guarantee the reliability of the results [28,29].

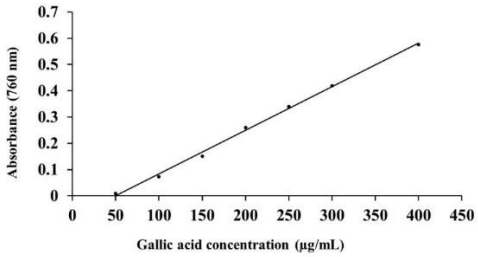

Figure 1. Analytical curve to determine total phenols content in the dry extract of guava leaf from ethanol 50% (DEGL-50) and ethanol 70% (DEGL-70) samples.

The TPC is the dry extract estimated spectrophotometrically by the Folin-Ciocalteu reaction [30–32]. The Folin-Ciocalteau reagent (FCR) consists of a mixture of

phosphomolybdate and phosphotungstate, whose basic principles are a redox reaction with the phenolic compounds available in the sample to form a blue-colored complex that absorbs radiation and allows it to be quantified by visible light spectrophotometry [33,34].

The FCR works by measuring the amount of analyte needed to inhibit oxidation of the reactive. However, the reaction occurs with other reducing substances that contain, for example, nitrogen such as guanidine and hydroxylamine, carotenoids, amino acids, sugars, and ascorbates. Faced with all the limitations, FCR is the adequate way to use the term Folin-Ciocalteau index, and it had been considered in the present study to estimate the TPC expressed in mg gallic acid.

In this study, the solvent, the time, and temperature of the extraction process have designed to decrease the presence of reactive organic acids in the extracts. Although the highest content of TPC has found in DEGL-50, Folin-Ciocalteau index of 0.57 (574.4 mg GAE/g), while the DEGL-70 sample had a Folin-Ciocalteau index of 0.42 (426 mg GAE/g) in relation to the dry extracts, it is needed consider that due to the highest water solubility of organic acids its content in DEGL-50 extract may be more than in DELG-70 extract. The study carried out by Seo *et al*. (2014) [11] TPC content of 185 mg GAE/g and 150 mg GAE/g in extracts prepared by maceration method with ethanol 50% and 70%, respectively. Other reports in the literature confirmed levels of 288.56 mg GAE/g on the dry extract obtained with ethanol 50% [26], and on the dry extract obtained with 70%, the TPC of 229.80 mg GAE/g were recorded [35].

3.3. In vitro antioxidant activity.

The formation of free radicals by the body under normal circumstances is inevitable, given that these compounds regulate cell growth, in addition to inhibiting the proliferation of viruses and bacteria [36]. Oxidative stress can damage the body´s cells, leading to a range of disease due to free radical that affect, for example, the central nervous system, accelerates the onset of cardiovascular disease, age-related vision decline, disorders autoimmune, inflammatory and cutaneous, degenerative genetic disease. Camarena-Tello *et al*. (2018) [6] report that the consumption of antioxidants significantly decreases the adverse effects caused by the excess of some reactive oxygen and nitrogen species in the human body.

DPPH (α, α-diphenyl-β-picrylhydrazyl) method has been widely applied to estimate antioxidant activity due to free radical scavenging and because it may be utilized in aqueous and nonpolar organic solvents. Besides, it can be used to examine both lipophilic and hydrophilic antioxidants; it is rapid, inexpensive, and simple.

Amongst all the available methods, the DPPH method offers the first approach for evaluating the overall antioxidant potential of an extract or other biological sources [37,38].

On mixing DPPH solution with a substance that can donate a hydrogen atom, it gives rise to the reduced form with the loss of violet color with absorption in ethanol solution at around 520 nm [23,39].

According to Morais-Braga *et al*. (2017) [27], the main phenolic compounds detected in guava leaf extracts are gallic acid, catechin, chlorogenic acid, caffeic acid, epicatechin, rutin, quercetin, kaempferol, and luteolin. These compounds play an important role in antioxidant activity, not only because of their ability to donate hydrogen or electrons but also because of their stable intermediate radicals, preventing the oxidation [40].

Based on the data obtained from this study, both the extracts were effective free radical inhibitor or scavenger, as well as a primary antioxidant that reacts with free radicals, which

may limit free radical damage occurring in the human body. The analysis of results by DPPH method showed that the greatest free radical scavenging was estimated for the DEGL-50 (70 %), while the DEGL-70 sample (63 %). This result shows that the extraction process used reached the greatest performance to isolate phenolics compounds, the same when compared to another extraction method reported in the literature, including the ultrasound-assisted extraction.

3.4. Fourier transform infrared spectroscopy (FTIR).

The FTIR spectrum of guava leaves and DEGL-50 and DEGL-70 samples were obtained using medium infrared (4000 – 400 cm^{-1}) and are shown in Figure 2 and Table 1. The presence of characteristic peaks was observed in the region of the functional groups (4000 – 1300 cm^{-1}), digital printing region (1300 – 900 cm^{-1}), and in the final region (900 – 400 cm^{-1}) of the spectra [41].

In the DEGL-70 spectrum, characteristic peaks were observed at 3383 cm^{-1} attributed to the hydroxyl stretch (OH) vibration; in the regions at 2927 cm^{-1} and 2856 cm^{-1}, they are attributed respectively to asymmetric and symmetric vibration of aliphatic stretching structures. In 1693 cm^{-1} corresponds to the elongation of the carbonyl group (C=O), and in the 1614 cm^{-1}, 1517 cm^{-1} regions are assigned respectively to the CH group and C=C elongation bands, typical of aromatic molecules.

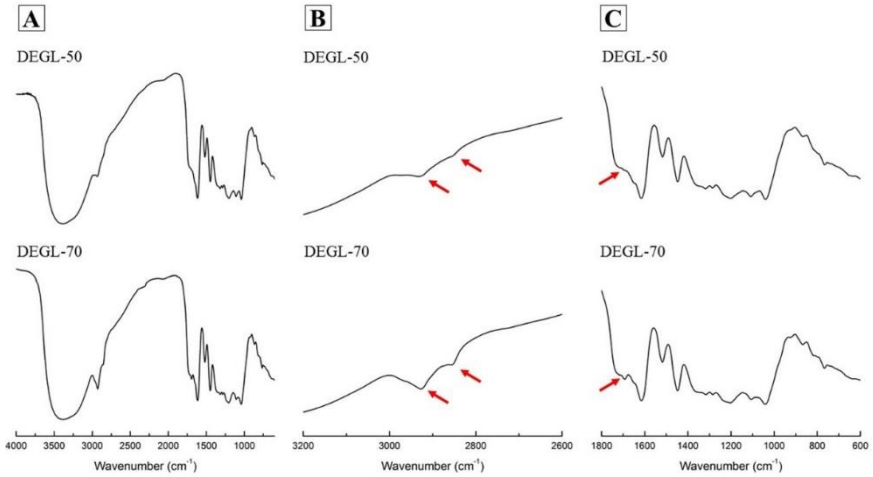

Figure 2. FTIR spectra of dry extract of guava leaves from ethanol 50% (DEGL-50) and ethanol 70% (DEGL-70) samples. The arrows on panel B and C indicate changes between the spectra of the samples.

The transmittance at 1446 cm^{-1} corresponds to the antisymmetric flexion in the CH_3 plane. The regions at 1203 cm^{-1}, 1107 cm^{-1} and 1041 cm^{-1} correspond to C–O stretching vibrations. Finally, the 767 cm^{-1} regions is attributed to out-of-plane conformations in CH [42–44].

The structural chemical conformation of the DEGL-50 and DEGL-70 samples recorded by the FTIR spectrum showed similar peaks. However, peak shifts and changes in transmittance intensity have been observed. In the DEGL-50 sample, in addition to the peak displacements, there were significant reductions in peak transmittance intensity in the regions of 2929 cm^{-1}, 2856 $cm^{-1,}$ and 1693 cm^{-1} (highlighted with arrows in Figure 2-B and C). The positions and assignments of the detailed peaks are listed in Table 1.

Table 1. Wavenumber assignments of FTIR spectra of dry extract of guava leaves from ethanol 50% (DEGL-50) and ethanol 70% (DEGL-70) samples.

Samples		Attribution
DEGL-50	DEGL-70	
Peak (cm^{-1})		
3383	3383	OH, stretch vibration
2931	2927	CH, asymmetric stretching vibration of aliphatic
-	2856	CH, symmetric stretching vibration of aliphatic
-	1693	C=O, stretching band of carbonyl
1616	1614	Aromatic CH bonds
1517	1517	C=C, stretching bands
1446	1446	Antisymmetric in-plane bending of CH_3
1201	1203	Stretching vibration of C–O
1109	1107	Stretching vibration of C–O
1039	1041	Stretching vibration of C–O
765	767	CH out-of-plane conformations

Through the data recorded by the FTIR, it was possible to verify changes in the spectra of the samples DEGL-50 and DEGL-70, confirming that the proportion of the solvent used in the extraction process influences the diversity of bioactive compounds extracted from guava leaves. The ethanol solvent, when used to extract bioactive substances, favors the extraction of compounds of intermediate polarity due to its reduced dielectric constant (Ɛr ~ 24.30 at 25 °C) when compared to water (Ɛr ~ 78.36 at 25 °C) [45]. The hydroethanolic solvents used in the study promote changes in the Ɛr, resulting in ~ 52 and ~ 40 for the percentages of 50 and 70%, respectively. Both the extractive capacity of phenolic compounds and the expression of antioxidant activity showed a direct correlation with this property. In addition, the observed changes in the spectra (Figure 2-B and C) attributed to the reductions in the asymmetric and symmetric vibration of the CH stretch of the aliphatic group and the C=O carbonyl elongation band, indicating the extraction of compounds with simpler structures for the DEGL-50 extract.

3.5. Determination of the aromaticity index by FTIR.

The FTIR technique provides reliable information for identifying the structure of chemical functional groups in complex mixtures, including quantification by absorbance or transmittance of the analyzed compounds [41,46].

The values of the aromaticity index (I1620/I2920) were calculated from the absorbance data presented in the FTIR spectra, obtaining values of 1.432 and 1.225, for samples DEGL-50 and DEGL-70, respectively (Table 2).

The aromatic index equation is a useful method for determining total phenolic compounds. Then, the total phenolics content was determined based on the aromatic index before used to evaluate the presence of aromatic groups in humic compounds [24] and to estimate aromatic properties in pedogenic and geological organics material [25] to estimate the aromatic characteristics present in humic substances of compounds and in the study presented by Dick *et al*. (2006) [25]

Table 2. Aromaticity index of dry extract of guava leaves from ethanol 50% (DEGL-50) and ethanol 70% (DEGL-70) samples.

Samples	Aromatic* and aliphatic# groups (cm^{-1})	Base H (cm^{-1})	Base L (cm^{-1})	Absorption intensity	Aromaticity index (AI)
DEGL-50	1616*	1849	1556	0.596	1.432
	2931#	2958	1921	0.416	
DEGL-70	1614*	1676	1558	0.673	1.225
	2927#	2999	2862	0.549	

The correlation between the values of total phenolic compounds determined by gallic acid equivalence (GAE) and the aromaticity index determined by FTIR, showed a high correlation, reaffirming that the sample DEGL-50 has the highest phenolic contents.

3.6. Hypothesis for the crosslinking reaction of glycosaminoglycan by "click chemistry".

Figure 3 shows the oxidative cleavage of the glycosidic group of sulfated glycosaminoglycan (ulvan), and crosslinking of the phenolic group.

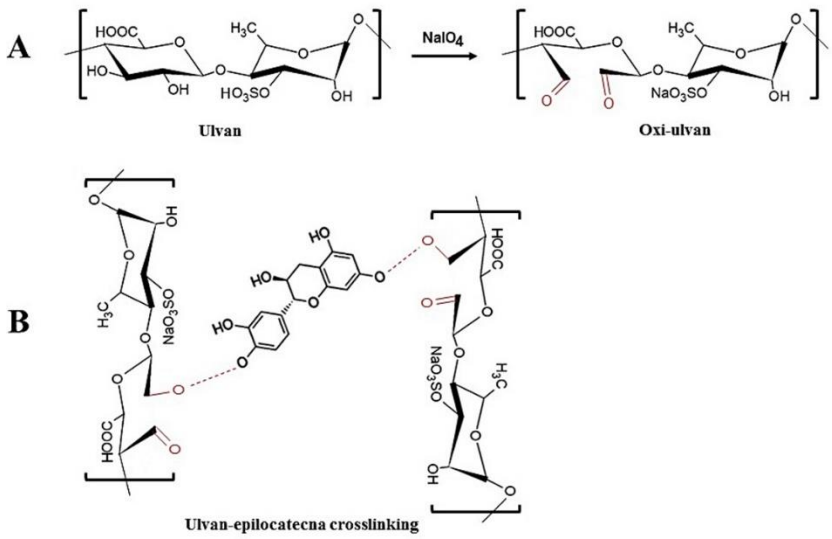

Figure 3. The hypothesis of glycosaminoglycans crosslinking by. A – Cleavage of the glycosidic group of the ulvan glucuronic acids; B – "click" reaction of epilocatecna for crosslinking of ulvan molecules.

4. Conclusions

The choice of the extraction method and experimental variables to obtain the dry extracts of the leaves of *Psidium guajava* Linn (var. *Pomifera*) were the most important factors to guarantee a higher yield. The ultrasound-assisted extraction technique, whose main effect in improving the process, is attributed to the acoustic cavitations produced in the solvent through the passage of an ultrasonic wave. These cavitations create shear stresses, causing the cell walls to rupture and allowing greater penetration of the solvent into the material matrix. Both hydroethanolic solvents showed a potential capable of obtaining dry extracts in 11% mass. The effect of the solvent dielectric constant had a direct influence on the content of total phenolic compounds, presenting a higher content for the DEGL-50 sample. The phenolic compounds present in both samples showed the ability to donate electrons and hydrogen atoms, with antioxidant activity of 70% and 63% (DEGL-50 > DEGL-70). The FTIR identified the main characteristic peaks of the samples, providing significant data on the chemical structure, determining the aromaticity index; this data set confirmed that the use of a hydroethanolic solvent (50%) increases the prevalence of aromatic structures in the extract; however, the extraction results in phenolic compounds with simpler structures. In conclusion, the results above provide a potential basis for the use of phenolic compounds extracted from guava leaves as crosslinking agents glycosaminoglycans (hyaluronic acid, chondroitin sulfate, ulvan) and proteins (collagen).

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Conflicts of Interest

The authors declare no conflict of interest.

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