Effect of the structure and some physicochemical parameters in the formation of the complex pectin-polyphenol and its impact on the hypoglycemic properties in a functional drink.

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Abstract:
The aim of this study is to determine the effect of the polyphenol structure (Procyanidin B2, (+)- Catechin and chlorogenic acid), pectin (high (HM), low methoxyl (LM)), the acidifying medium (citric acid and malic), pH (3 and 4) and the presence of ions (KCl) on the formation of pectin-polyphenol complex in a model solution system that simulates a functional beverage matrix; and the impact of the formation of this complex in the biological effect of the beverage. The evaluation of the formation of pectin-polyphenol complex was performed by determining the% inhibition of DPPH radical and free polyphenols profile by HPLC in model solutions. The evaluation of the biological effect was performed by postprandial testing in mice Balb/c which were induced hyperglycemia with maltose. The results were analyzed by analysis of variance (ANOVA) with a 95% confidence. It was determined that the physicochemical parameters such as pH, the presence of ions and type of acidifying if they have a significant effect on the formation of pectin-polyphenol complex, and that the impact of these parameters depend on both the functional groups of the polyphenol as pectin that are in solution. Biological tests show that it is possible to determine the impact of pectin-polyphenol complex functional properties of a food, this allows us to lay the groundwork for the development of new functional foods where it is relevant to interactions with other nutraceuticals components of a food matrix.

Keywords: Physicochemical interaction, Postprandial testing, Hypoglycemia, Polyphenol, Pectin.

Introduction

In the food science, the physicochemical interactions between the different ingredients of a food dictate are desirable, due to promote stability, both physicochemical level as a microbiological level, as well as desired sensory properties (Buchweitz et al. 2013). However, the importance of these interactions in food has taken a new turn, due to the recent interest in the development of *functional foods*, they are those who, in besides satisfying the basic nutritional needs, provide different health benefits or reduce the risk of chronic diseases (Landete 2012; Khan et al. 2012; Álvarez et al. 2012). The biological effect of these foods is because certain phytochemicals, also called *nutraceuticals*, its biological effect has been scientifically proven in the individual ingredient, both in-vivo and in vitro. Examples of such phytochemicals are polyphenols and pectin; shown to have a

significant impact on the control or prevention of certain diseases such as in the case of hyperglycemia (Becerra Jiménez and Andrade Cetto 2012; Quiñones, Miguel and Aleixandre 2013; Padayachee, A. et al. 2012; Galati et al. 2002). In Mexico products formulated with these nutraceuticals may be of great importance, because diabetes, is the second place of death from disease nationwide in 2012 (Instituto Nacional de Estadística y Geografía 2014). *Opuntia ficus Indica* cladodes (nopal) is a source of pectin, underused in modern times but widely used in traditional Mexican medicine since pre-Hispanic times (Becerra Jiménez and Andrade Cetto 2012; Butterweck et al. 2011; Andrade Cetto and Wiedenfeld 2011). As a source of polyphenols for the design of this type of food avocado seed may be an interesting option, considering that besides, is a waste byproduct of avocado industry. Studies have proven the presence of polyphenols as catechins, procyanidins, anthocyanins, and tannins (Ramos Jerz 2007; Kosińska et al. 2012; Arukwe et al. 2012; Ramos Jerz et al. 2013). In a previous work, a soft drink was elaborated with aqueous extracts rich in polyphenols from seed Hass avocado (*Persea americana* Mill cv Hass) and nopal (*Opuntia ficus-indica*), which has proved to have a hypocholesterolemic and hypoglycemic effect on BALB/c mice, this provides the basis of this drink as a functional food. However, to scale the production of this beverage, at industrial level, it is important to know the effect they might have different additives that could be added for attaining adequate sensory characteristics, or to stabilize the beverage and prolong shelf life, so far, to have the domain of the formulation and process of this drink, it is essential know the factors that determine the physicochemical interactions between the components that make up the matrix of the beverage and nutraceuticals, and then determine if they can prevent that nutraceuticals could be completely absorbed by the body, to be shortly available or, protect polyphenols from degradation and potentialize the biological effect (Michalski et al. 2013; Landete 2012), and thus exploit the full potential of a functional food. Until now little is known about the interactions between pectin and polyphenols, however we know that when these compounds are contacted, rapidly form the complex pectin-polyphenol which is stable by non-covalent interactions, such as bridges hydrogen, ionic interactions and hydrophobic interactions. Such interactions are of different nature and dominance or prevalence of one or the other, depending on factors such as the structure (conformational units, functional groups and stoichiometry), degree of polymerization and some physical and chemical characteristics of the medium (pH, ionic strength, concentration of other components), among other (Le Bourvellec and Renard 2012; Watrelot et al. 2013; Sivam et al. 2012; Buchweitz et al. 2013). The aim of this study was to determine the effect of pH, presence of ions, polyphenol structure and degree of methoxyl pectin in the pectin-polyphenol interactions, which generate lower or higher availability of free polyphenols respectively, also the hypoglycemic effect of beverage by a biological test and thus in this way, indirectly infer the impact of physicochemical interactions on the availability of the bioactive molecules in the drink. The concentration of polyphenols in model solutions were established based on a preliminary analysis of the soft drink (made with aqueous polyphenolic extracts from Hass avocado seed and aqueous extract of nopal pectin, which has a patent pending), for measuring the amount of some polyphenols of interest.

Methods and Materials

Solvents and reagents.

Water and methanol, both HPLC grade, acetic acid, chlorogenic acid (CA), Procyanidin B2 (Pr), (+)-catechin (C), citrus pectin of high methoxyl (HM) (≥85%) and low grade methoxyl (LM) (34%), DPPH (1, 1-diphenyl-2-picrylhydrazyl), acarbose and maltose were obtained from laboratories SIGMA-ALDRICH, the Malic and citric acid and KCl FERMONT laboratory.

Animals

Twelve eight-week-old male BALB/c mice (25 ± 3g) were purchased fromthe Zooterio of theUniversity of Guadalajara. The mice were fed Standard Diet 20128 Tekland and water. They were kept at room temperature, under a 12 hours cycle of light and dark, at 22°C. Animals were handled following the animal care guidelines in accordance with regulations enacted by the Federal Government of Mexico (NOM-062-ZOO-1999 and NOM-033-ZOO-1995).

Preparation of the model solutions systems

The concentration of polyphenols was 25 ppm chlorogenic acid, 25 ppm (+)-Catechin and 25 ppm Procyanidin B2, 0.003gr / ml of high methoxyl pectin or low methoxyl, the pH was adjusted to 3 to 4 using citric acid or malic acid, and 0.01M KCl. Each solution prepared with deionized water. Polyphenols solubilization was accomplished by stirring with a magnetic bar and allowed to stand in the dark for 20 minutes before measurements.

Determination of the % inhibition of DPPH radical (%I).

This determination was done according to the technique reported by (Rodríguez-Carpena et al. 2011). In each well of the microplate was added 33µL the model solution and a solution 200µL $6x10^{-5}$M of DPPH radical (80% methanol and 20% distilled water). Samples were allowed to stand for 20 minutes in the dark and then were read on a microplate spectrophotometer at 217nm XMARK.

The results are reported using the following equation:

%I= ((Blank absorbance-Sample absorbance)/Blank absorbance) x100.

Determination qualitative and quantitative of free polyphenols.

Quantification was performed by High Liquid Chromatography (HPLC) on a Varian ProStar team with UV detector a Whatman C-18 column 4.6mm x 250mm.La elution by gradient, starting with 85% water acidified and acidified methanol 15% and ends at 40% and 60% water acidified acidic methanol, each at 1% of acetic acid. Flow was kept constant at 0.4 ml / min for 50 minutes. Identification was made based on the times of retention and verification standards, quantification was performed using a calibration curve.

Evaluation of the biological effect

The evaluation of the biological effect was performed by postprandial testing in mice Balb/c. Were formed Four groups of six mice each were used:

- Negative control, to which water was administered.
- Positive control, which was administered a solution of acarbose (3mg/kg mouse).
- Group A, which was administered with the model solution that presented higher concentration of free polyphenols.
- Group B, which was administered a solution that present the lowest concentration of free polyphenols.

After 18 hours of fasting, all the groups were administered a maltose solution (4g/kg mouse) to raise the levels of blood sugar, immediately after, all the different groups were administered a second solution (water, acarbose and the model solutions with a higher and

lower concentration of free polyphenols) so, indirectly determine the impact of physicochemical interactions on the availability of the polyphenols. After 30 minutes, a blood sample from the tail of each mouse was taken and analyzed by ACCU-CHEK Performa kit. The results presented are increased postprandial glucose, obtained by the difference between postprandial blood sugar (gr/dl) - basal blood sugar (gr/dl).

Statistical method.

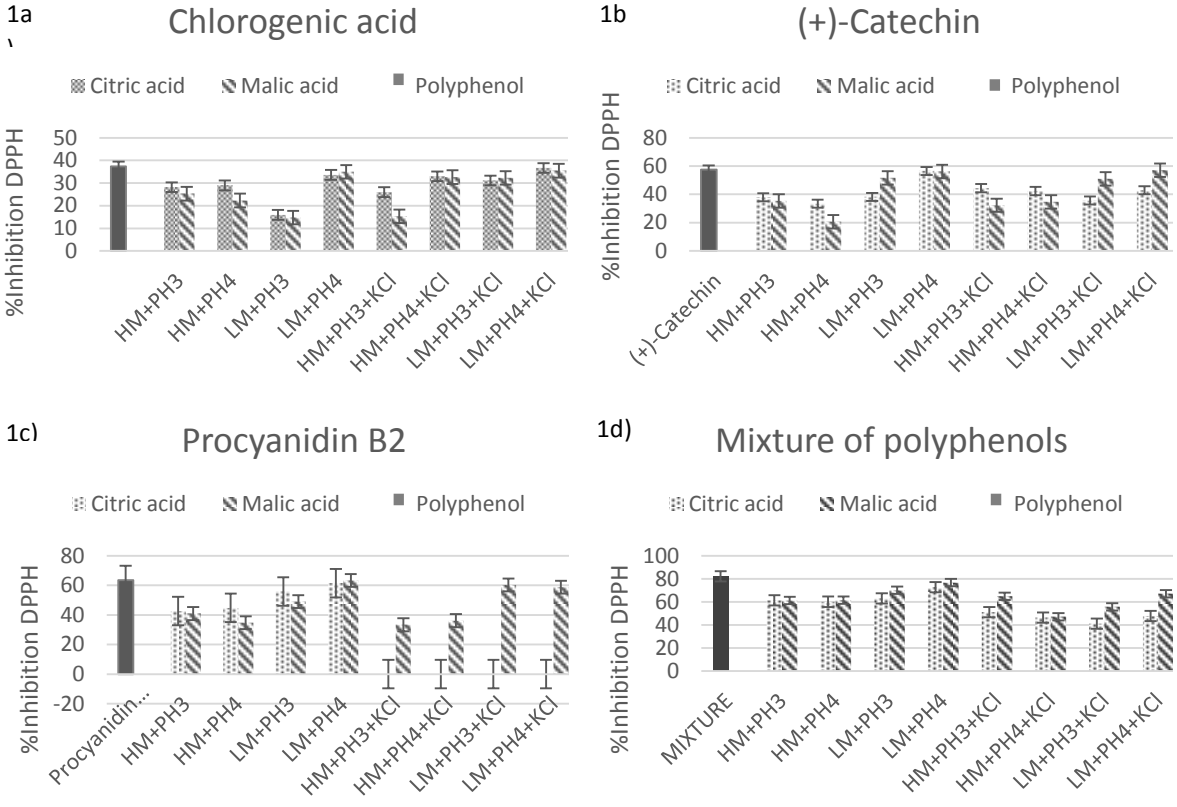
The results were analyzed using an Analysis of Variance (ANOVA) with STATGRAPHICS Centurion XVI software. To determine the availability of polyphenols in model solutions, the values obtained from the response variables of% DPPH radical I and quantification of phenolic profile were analyzed. For determining the biological effect, the results of postprandial sugar levels of the mice were analyzed.

Results

Determination of the % inhibition of DPPH radical (%I).

By this test is possible to infer that the higher the (%I) there will be a greater amount of free polyphenols therefore less interaction, and vice versa. The figure 1 shown the results of the determination of %I in individual polyphenols as a mixture of polyphenols.

Figure 1, test results of %I of the different model solutions.



Where: HM, LM (High and Low methoxyl pectin), pH3 and pH4 (pH of the solution), KCl (the presence of ions)

In Figure 1a, we see how the %I of the chlorogenic acid solutions (CA) at pH 3 with low methoxyl pectin (LM) is significantly lower than other solutions of CA, indicating that

there is less free radical CA to reduce DPPH, therefore it can be inferred that in these conditions is favored interaction pectin-polyphenol, on the contrary, it is noted that solutions to pH4 with LM have higher% I, indicating that there is a greater amount of free polyphenols, this may be due to the CA with a pKa of 4.3 (Padayachee, A et al. 2012), as the galacturonic acid with pKa 2.9 (Le Bourvellec, Guyot and Renard 2004), are ionized with negative charge (COO-) at pH4, generating the repulsion between the two groups, whereas CA at pH 3 is not ionized, which favors ionic interaction with LM. In addition, the statistical analysis also shows that the interaction HM with CA also performed, but it is not influenced by the pH, which can be caused by the esterification of the galacturonic acid, which prevents ionization of the chain of homogalacturonan, however, this interaction is enhanced significantly by the presence of KCl, indicating that the interaction between these compounds is carried out by hydrophobic interactions. Regarding the effect of the acidifying medium on the formation of pectin-polyphenol complex, only significant difference is observed in the solution of pH 3 + KCl, where greater interaction exists in acidic medium with malic acid, this may be due to this acid unlike citric acid has the ability to interact with ions (K^{+2}), which may favor the strengthening of the network of interactions of the matrix solution.

Figure 1b shows the %I of the solutions of (+)-Catechin (C), in this is observed that the interaction of HM and C to pH 4 with malic acid is the most intense. The statistical analysis shows no matter the acidifying medium C has higher affinity for HM than by LM. what can occur because HM can generate hydrophobic interactions a pH close to 4 (Fennema R. 2000) among the heterocyclic di-pyran rings of C and of hydrophobic pockets of HM. The interaction of C by HM is enhanced by the presence of KCl, this may be due to the presence of salts in solution can promote hydrophobic interactions between the components of the solution, this is because the ions changes the polarity of the solvation shell of water formed around the nonpolar fractions of the molecules of both C and HM, has been reported that the ions with higher charge density and least polarizability, forming stronger complexes with hydration solvation shell, increasing its distance from hydrophobic surfaces, generating stability increasing the entropy of the system (Mancera 1998), however, this effect is not observed in solutions of malic acid, this may be due to the chelating ability of the acid, which traps ions complexing preventing these hydration. Furthermore, in figure 1c can be observed as procyanidin B2 (Pr) appears to have similar behavior to the (+)-catechin, due to the more interaction is generated with HM to pH4, indicating the presence of hydrophobic interactions, however it was observed that the solutions of malic acid in the presence of KCl, regardless of pH and pectin, do not exhibit %I. As discussed above, the malic acid is a strong chelator of ions, but so is Pr, this can lead to the formation of a network between malic acid and Pr which would be maintained by salt bridges, on the other hand, citric acid not have shown the ability to chelate ions, so they may be free procyanidins can inhibit the DPPH radical.

In Figure 1c it is noted that the model solutions with the mixture of the three polyphenols, had the same behavior as in the solutions of C and Pr, this is because these polyphenols generate the highest antioxidant capacity in the system, so that the% I depend on whether they are free or not. In this case the statistical analysis showed that the solution which was lower free polyphenols was low methoxyl pectin, pH3 and KCl with citric acid

(pH3+LM+KCl), and lower interaction or greater availability is the model solution low methoxyl pectin to pH4, however in this case, the type of acid did not affect the interaction.

Determination of free polyphenols.

With this test it was possible to confirm the observations made in the determination of the% I, since the results of the solutions of larger and lower interaction identified above coincide with those of this test. In Figure 1 are shown some of the chromatograms obtained for solution with the mixture of polyphenols, in them it can be seen that the chlorogenic acid was the compound less interacted with pectin, regardless of the pH, the presence of KCl or the type of pectin; followed by the (+)-Catechin and finally procyanidin B2. This result makes us think that there is competition between polyphenols by the active sites of pectin, due to is the Procyanidin B2 which form interactions more easily, this may be due to the fact that this has a greater number of binding sites capable to form more stable interactions.

Table I, chromatograms of the different solutions with mixtures of polyphenols.

CITRIC ACID						MALIC ACID					
Solution	**Polyph.**	**Concent.**	**Solution**	**Polyph.**	**Concent.**	**Solution**	**Polyph.**	**Concent.**	**Solution**	**Polyph.**	**Concent.**
HM+pH3	AC	9.5 ± 0.28	**LM+pH3**	AC	24.36 ± 0.04	**HM+pH3**	AC	5.65 ± 0.2	**LM+pH3**	AC	7 ± 0.14
	C	ND		C	7.92 ± 0.16		C	ND		C	ND
	Pr	ND		Pr	0.64 ± 0.2		Pr	ND		Pr	ND
HM+pH4	AC	12.26 ± 0.19	**LM+pH4**	AC	20.55 ± 0.21	**HM+pH4**	AC	9.75 ± 0.07	**LM+pH4**	AC	3.56 ± 0.15
	C	5.1 ± 0.14		C	8.05 ± 0.21		C	5.65 ± 0.49		C	ND
	Pr	ND		Pr	ND		Pr	ND		Pr	ND
HM+pH3 +KCl	AC	13.27 ±0.04	**LM+pH3 +KCl**	AC	22.75 ± 0.07	**HM+pH3 +KCl**	AC	20.8 ± 0.28	**LM+pH3 +KCl**	AC	24.75 ± 0.22
	C	ND		C	7.55 ± 0.23		C	7.75 ± 0.21		C	4 ± 0.14
	Pr	ND		Pr	ND		Pr	ND		Pr	ND
HM+pH4 +KCl	AC	ND	**LM+pH4 +KCl**	AC	ND	**HM+pH4 +KCl**	AC	17.45 ± 0.18	**LM+pH4 +KCl**	AC	ND
	C	ND		C	ND		C	6.05 ± 0.12		C	ND
	Pr	ND		Pr	ND		Pr			Pr	ND

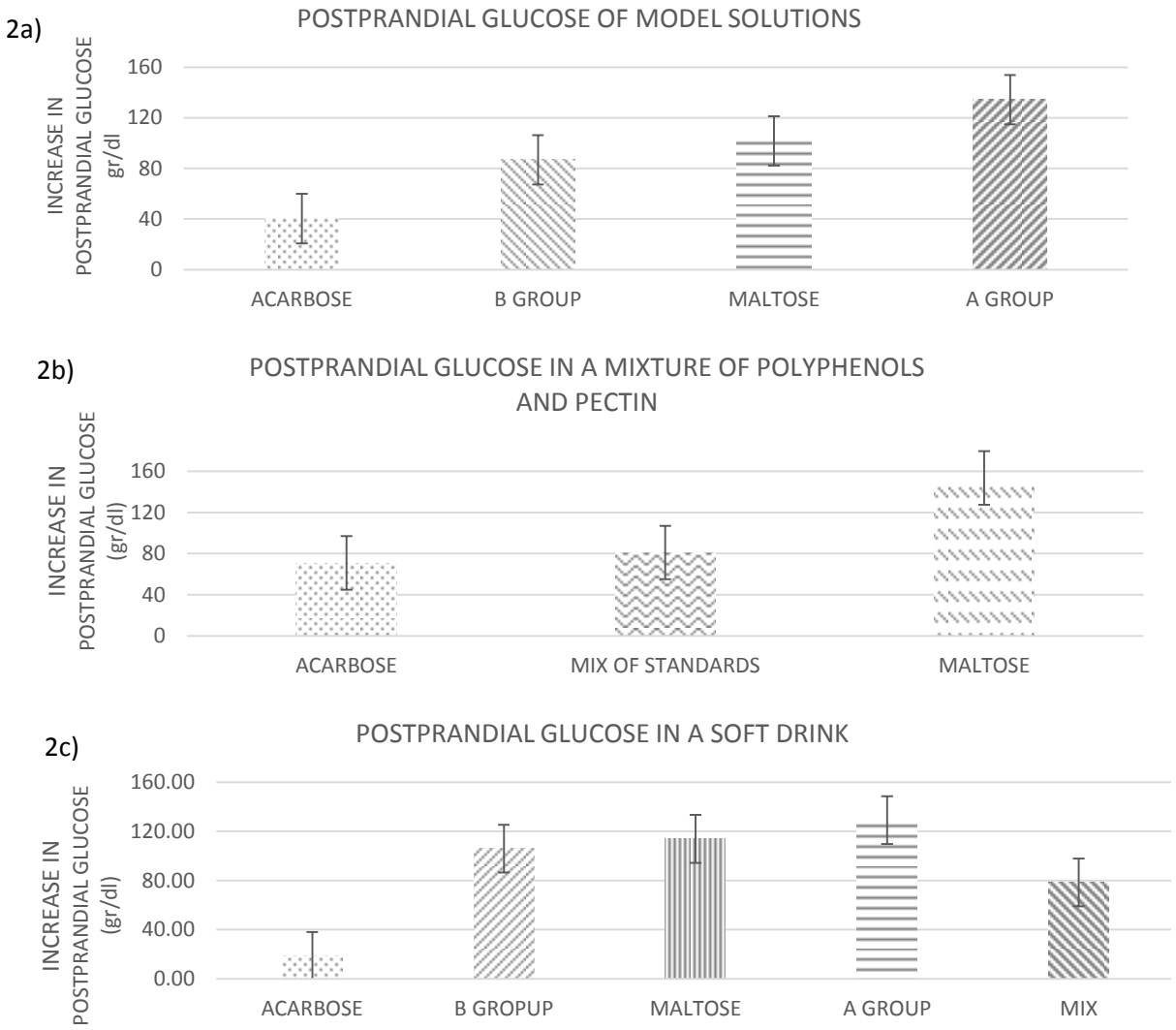
Where: HM, LM (High and Low methoxyl pectin), pH3 and pH4 (pH of the solution), KCl (the presence of ions), AC (chlorogenic acid), C ((+)-Catechin), Pr (Procyanidin B2)

Evaluation of the biological effect

In Figure 2 the results of biological testing are shown, in Figure 2a shown as the acarbose presents the lowest increase in postprandial glucose, followed by the solution having lower availability of polyphenols (Group B), however statistical analysis indicates no significant difference between the groups treated with the model solutions and the negative control (maltose), however at repeat of the experiment a tendency was observed, the solution with less availability of polyphenols tend to avoid the increase of glucose blood more effectively than the solution having greater availability of polyphenols (Group a). Subsequently a test with a model solution which contained only the mixture of polyphenols and pectin, this solution generated a very interesting result, since it reduces the postprandial rise in glucose at the same level that acarbose, which makes us think that possibly some of the components of the model solutions is interfering with the mechanism of pectin and polyphenols to

prevent absorption of glucose in blood (Figure 2b). Finally, they were administered to the mice soft drink made by aqueous extracts rich in polyphenols from Hass avocado seed, aqueous extracts of nopal pectin, sucrose, sodium benzoate and the conditions that generate highest (Group A) and lower (Group B) availability of polyphenols. This trial confirmed the observations with model solutions, because even though there is no statistically significant difference between the group administered with the mixture of extracts from seed and nopal, group A, group B and group maltose, the soft drink with reduced polyphenol availability still greater hypoglycemic effect than the most widely available soft drink (Figure 2c), in this case it is worth emphasizing that although the mice in groups a and B, both the solution consumed maltose, and sucrose from the soft drink did not show glucose levels higher than those of maltose ingested alone group, indicating that there is indeed a hypoglycemic effect by the functional beverage.

Figure 2, Results of biological testing.



Where: acarbose (positive control), maltose (negative control), group A (higher availability of polyphenols), group B (lower availability of polyphenols), mixture of standards (Ac Chlorogenic, catechin, Procyanidin and low methoxyl pectin.) Mix (Agucate seed extracts and extracts of nopal)

Discussion

Chlorogenic acid, (+)-Catechin and procyanidin B2 have a higher affinity for the more complex solutions of the high methoxyl pectin, complexation between those polyphenol and high methoxyl pectin appears to be favored by hydrophobic interactions. The interaction with low methoxyl pectin also performed, however, due to the chemical nature of each polyphenol the complexing mechanism takes different paths. Chlorogenic acid interaction with Low methoxyl pectin seems to occur mainly by ionic interactions and is primarily affected by the pH and the presence of KCl, whereas the interaction of the (+)-catechin, procyanidin B2 and the same pectin it is maintained mainly by hydrophobic interactions and is affected by the presence of KCl. Even research is lacking in this regard, however at this point we are able to determine the combination of factors that allow us to have greater or lesser availability of polyphenols.

Concerning the biological assay, it was determined that the concentration of polyphenols with which we worked is enough to produce an effect on postprandial glucose levels, however, also observed that the acid medium, the pH or the presence of ions may impair the biological effect of pectin or polyphenols, as their biological activity is decreased in the presence of these agents. Yet it was possible to conclude that the solution with the lower availability of polyphenol has greater impact in postprandial glucose levels compared to the higher availability of polyphenols. It is possible that pectin-polyphenol interaction prevents the absorption of some polyphenols in the stomach and reach the small intestine intact, which subsequently undergo conjugation reactions by intestinal and hepatic enzymes (Fraga et al. 2010; Scalbert et al. 2002), and then are transported through the intestinal epithelial by protein SGLT-1 (Németh et al. 2003), which belongs to a family of membrane proteins coupled to Na +, which also specializes in the active transport of glucose. So there is the possibility that, coupled with the possible effect of barrier against glucose of the nopal pectin (Andrade Cetto and Wiedenfeld 2011; Butterweck et al. 2011), polyphenols prevent the passage of glucose into the bloodstream by competing for the active sites of the SGLT-1 proteins.

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