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Effect of pectin on the interactions among phenolic compounds determined by antioxidant capacity

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ABSTRACT

Antioxidant capacity of the phenolic compounds depends on their chemical structures and interactions between them. Twenty binary combinations between four phenolic acids (gallic acid, coumaric acid, chlorogenic acid, caffeic acid) and four flavonoids (catechin, quercetin, epicatechin, and rutin) were analyzed to evaluate the effect of their combinations by DPPH and FRAP methods. Isobolograph analysis and combination index (CI) were used for DPPH, and the percentage of differentiation for FRAP method to evaluate the effect of the PC binary combinations (synergism, additive or antagonism). Chlorogenic acid presented the highest inhibition of DPPH, while coumaric acid did not present any DPPH inhibition. Gallic acid and catechin showed better ferric reducing capacit. The antioxidant capacity increased when pectin is mixed with individual PC. The isobologram method shows that the PC binary combinations presented additive or antagonistic effects in the DPPH method. A synergistic effect on DPPH radical inhibition and FRAP method was presented in most of the combination of phenolic compounds with pectin. The antioxidant capacity depends on the type and the location of hydroxyl, and the type of phenolic compounds.

1. Introduction

Phenolic compounds (PC) are natural non-energetic compounds biosynthesized by plants that include in their structure at least one aromatic ring and a hydroxyl group [1]. Depending on the number of aromatic rings, more than 8000 different PC have been identified [1,2]. The main PC groups, depending on the carbon skeleton, include C6-C1, hydroxybenzoic acid; C6-C2, C6-C3, hydroxycinnamic acids; C6-C2-C6, stilbenes; C6-C3-C6, flavonoids; $(C6\text{-}C3\text{-}C6)_2$, biflavonoids (agathisflavone); and condensed tannins $(C6\text{-}C3\text{-}C6)_n$. PC present various biological activities such as antioxidant capacity that is determined by their chemical structure, so they have redox properties by two main mechanisms: hydrogen atom transfer and electron transfer [3]. The result of these mechanisms generates the ability to trap free radicals and the effect to chelate transition metals [1,3]. There are empirical mathematical models that have been proposed that analyzed the biological effect of the interactions between drugs, establishing if these interactions produce synergistic, additive or antagonistic effects [4,5]. Among the most used models are isobologram model, combination index and differentiation percentage [6].

Iisobolograms are, for their simplicity, the most used in practice, since they are graphical representations in a coordinate axis of iso-effective doses of each of the compounds used individually and of their combination [6–8]. These models make it possible to evaluate whether additive, antagonistic or synergistic activity exists. Synergistic activity is when the combination of two compounds is more effective compared to the sum of the individual activity of both compounds individually. Antagonism consists when the combination of two compounds is less effective than each compound individually, and higher doses will be needed to produce the same effect, and finally, an additive effect is considered when two compounds do not interact, and the observed effect corresponds to the sum of each individual doses [7].

On the other hand, some studies have shown that some components, such as dietary fiber (DF), that bind to PC can interfere with antioxidant capacity. Pectins are a complex group of heteropolysaccharides with different degrees of esterification, which may contain 200–1000 units of galacturonic acid linked by glycosidic α 1-4 bonds, which are present in the primary cell wall and in the mid-laminates of the parenchymal cells of fruits and vegetables,

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where they are frequently associated with other components such as cellulose, hemicellulose and lignin, and are responsible for the firmness of these products [9,10]. Pectins can be distinguished into two main classes: pectinic acids, which have a small portion of their galacturonic acids as methyl esters, and pectic acids, which contain only esterification-free galacturonic acid molecules. This type of structure confers some degree of interaction with other food components, such as PC [11]. Therefore, DF has the ability to trap these compounds depending on the degree of esterification, preventing them from performing their antioxidant activities [11]. In order to evaluate the interaction of the PC, as well as their possible interaction with DF, the objective of this study was to carry out a study to evaluate the synergistic effect of the binary combination of the PC on antioxidant capacity, determining the combination index by the isobologram method for the DPPH method and determining the percentage of difference for the FRAP method.

2. Methodology

2.1. Chemicals

Gallic acid (GaA); coumatic acid (CoA), chlorgenic acid (ChA), caffeic acid (CaA), catechin (Cat), quercetin (Que), epicatechin (ECa), and rutin (Rut), chlorhydric, acid, methanol, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric chloride hexahydrate, and 2,4,6-Tris(2- pyridyl)-1,3,5-triazine (TPTZ) were purchased from Aldrich-Sigma (St. Louis, MO, USA).

2.2. Preparation of PC solution

GaA; CoA, ChA, aA, Cat, Que, ECa, and Rut (Fig. 1) were dissolved in methanol (80% v/v) at a 10 mM concentration, and stored in amber flasks at 5 °C.

2.3. Antioxidant capacity of phenolic compounds

2.3.1. Determination of DPPH radical scavenging capacity

The assay is based on the loss of violet color of 2.2-diphenyl-1-picrylhydrazyl (DPPH•) solution when reduced by PC, was carried out as described by Alvarez-Parrilla [12]. For the assay, 25 μL PC solution was mixed with two hundred microlitres of the 230 μM DPPH solution in a microplate (BioRad XMark Plus, USA). After 15 min incubation, the absorption change was measured at 517 nm and DPPH reducing capacities was also expressed in μM. 600−16.25 μM working solutions of PC solution were used for calibration. The percentage of DPPH• inhibition (%$DPPH_{Inh}$) was calculated according to equation (1):

$$\%Inh = \left(\frac{(DPPH - PC)}{DPPH}\right) \times 100 \quad (1)$$

where %Inh is the percentage inhibition of the DPPH radical, DPPH is the absorbance of DPPH radical solution (230 μM) without PC, PC is the phenolic compound solution. The results were expressed as IC_{50}, inhibitory concentration of the PC needed to reduce the molecules of DPPH radical by 50% (μM), calculated from dose−response relationship using median-effect equation which in this article was used to describe concentration−inhibition relationship according to following equation (2) [19]:

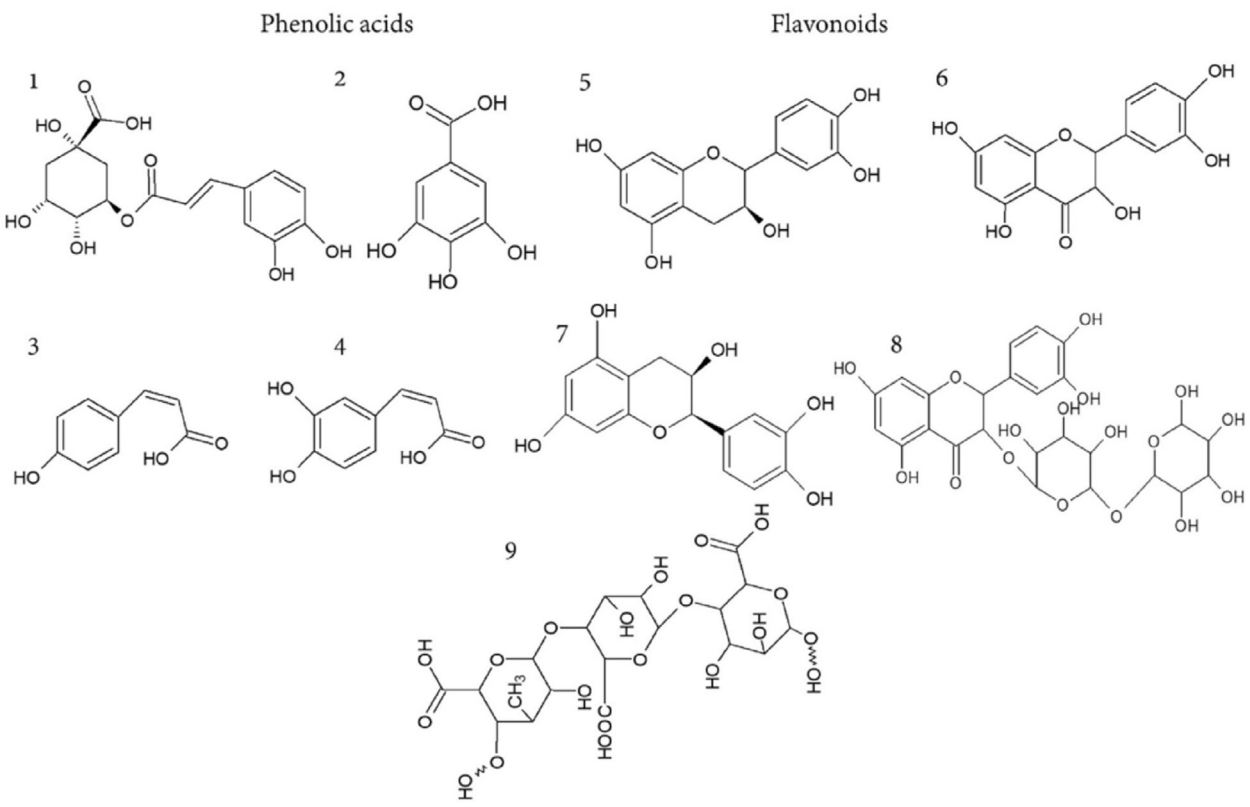

Fig. 1. Phenolic compounds used in the antioxidant capacity. 1) Chlorogenic acid; 2) Gallic acid; 3) Coumaric acid; 4) Caffeic acid; 5) (+)-Catechin; 6) Quercetin; 7) Epicatechin; 8) Rutin; 9) Pectin.

$$DPPH_{Inh} = \frac{1}{1 + \left(\frac{IC_{50}}{c}\right)m} \quad (2)$$

where c is the concentration of the tested solution, and m is the coefficient signifying the shape of the curve. The IC_{50} and m, from media-effect equation, were calculated by non-linear regression method.

2.3.2. Ferric reducing antioxidant power (FRAP)

FRAP assay was performed as previously described by Ref. [13]. FRAP is based on the ability to reduce yellow ferric tripyridyltriazine complex (Fe(III)-TPTZ) to blue ferrous complex (Fe(II)-TPTZ) by electron-donating PC in an acidic medium, which is measured as an absorbance change of ferrous TPTZ complex. For this analysis the Trolox (0.13–0.08 mM) were used as standard and the IC_{50} values of each PC obtained from DPPH method was used for this method. The FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), TPTZ solution (10 mM TPTZ in 40 mM HCl) and $FeCl_3{}^*6H_2O$ (20 mM in water solution) in 10:1:1 v/v ratio. After incubating for 30 min at 37 °C in a water bath, the absorbance readings were taken immediately at 593 nm. The FRAP value is expressed as μM of Trolox equivalent.

2.4. Antioxidant capacity of the binary interactions among phenolic compounds

PC were combined in binary form, having twenty-one interactions. From the IC_{50} of each PC three ratios levels were evaluated: 30A:70B%: 50A:50B% and 70A:30B%, respectively. Results obtained were analyzed by the Isobolographic analysis, according to Tallarida [14], to evaluate the synergistic, additive or antagonistic effect. The IC_{50} of each individual PC is plotted as X and Y axes and the calculated IC_{50} value for different concentration combinations is calculated and plotted to observe if this values are above (antagonistic effect) or below (synergistic effect) the line plotted with both IC_{50} values. To corroborate the isobologram results, the interaction of the binary PC combination was determined by the combination index (CI, Eq. (3)) according to Chou [15]:

$$CI = \left(\frac{IC50_{ab/2}}{IC50_a} + \frac{IC50_{ab/2}}{IC50_b}\right) \quad (3)$$

where IC_{50a} and IC_{50b} are the IC_{50} values obtained for pure PC, while $IC_{50ab/2}$ is the concentration of individual compound in the mixture that cause 50% of inhibition. The obtained CI values indicate an additive (IC = 1), synergistic (IC < 1) or antagonistic (IC > 1) effect.

In the case of FRAP method, the percentage of differentiation (% D) [16] was determined by the following equation (4):

$$Difference\ (\%D) = \left[\frac{C_{ab} \times 100}{PC_a + PC_b}\right] - 100 \quad (4)$$

where C_{ab} is concentration obtained for PC combined. PC_a and PC_b is the concentration of individual PC, respectively. %D is the potential additive, synergistic or antagonistic effect.

2.5. Effect of pectin on the antioxidant capacity of phenolic compounds

Pectin was added in the binary combination of PC to analyze the effect on the antioxidant capacity described above. Three aqueous pectin solutions (5 mg/mL, 10 mg/mL, 30 mg/mL) were prepared to analyze individually, with the individual PC and the binary combination of the PC. The concentrations taken into account for both studies were 50% of the IC_{50} concentration of each PC, previously obtained. IC and %D were determined from equations (3) and (4).

2.6. Statistical analysis

Statistical of FRAP and DPPH values of individual PC, combined and interactions between pectin were determined by performing the analysis of variance (ANOVA), followed by a least significant difference test at 95% confidence level and the Tukey test were performed using Statistica® software (StatSoft Inc., Tulsa, USA), 7.0 version. The data are presented as mean ± standard deviation (SD). Three samples (n = 3) of each experiment were analyzed, and all assays were performed in triplicate.

3. Results and discussion

3.1. Determination of DPPH radical scavenging capacity

The IC_{50} of the DPPH radical scavenging capacity for each PC are shown in Table 1. This table shows a comparison between previously reported DPPH and FRAP values with our results, where all of the analyzed compounds are within the range shown in the table. In this table shown that CaA and Que presented a better inhibition of the radical DPPH, followed by GaA and ChA. Flavonoids had a greater ability to inhibit the DPPH radical than phenolic acids, because the transfer mechanisms of hydrogen atoms and the dominant mechanism that are determined by the properties and chemical structure of the PC, as well as the bond dissociation energy and ionization potential are factors that determine the mechanism and effectiveness of the PC [27].

However, our study differs from this theory because CaA has a better inhibitory capacity than Cat and Rut. Previous studies have shown that CaA shows an exceptionally high antioxidant capacity due to the electrochemical behavior of the two hydroxyl groups that are in the *ortho* position on the ring (Fig. 1), which suggests

Table 1
Determination of IC_{50} by the DPPH method and concentration of antioxidant capacity by the FRAP method of the phenolic compounds.

Phenolic compounds	DPPH (IC_{50}, μM)	Reported DPPH (IC_{50}, μM)†	Reference	FRAP (μM)	Reported FRAP (μM)	Reference
GaA	125.89 ± 4.5^{c}	54.5–264.2	[17,19,20,24]	42.41 ± 0.63^{b}	49.5–861.3	[18,20]
CoA	NA	1–43.9	[17,20,23]	38.48 ± 1.03^{c}	0.0–38.5	[20]
ChA	137.40 ± 11.9^{b}	132.65–1278	[21]	37.91 ± 0.31^{c}	37.00–1278	[18]
CaA	119.17 ± 23.3^{d}	16.6–1641.3	[17,20,23]	$37.91 \pm \pm 0.46^{c}$	2.19–1641.3	[18,20]
Cat	234.77 ± 17.00^{a}	5.4–251.4	[19,20]	$56.43 \pm \pm 0.94^{a}$	15.8–215.4	[20,24]
ECa	143.76 ± 36.6^{b}	178.8	[20,24]	43.22 ± 0.77^{b}	10.8–145	[20,24]
Que	116.97 ± 4.2^{d}	115.5–498.5	[24]	42.20 ± 0.67^{b}	29.2–864.7	[18,21,24]
Rut	143.76 ± 14.9^{b}	117.3–372.3	[19,25,26]	31.92 ± 0.79^{c}	11.2–704.7	[19,25]

Values are the means of three replicates ± standard deviation (SD). Values with different lower letters are significantly different between phenolic compounds. GaA: gallic acid; CoA: coumaric acid; ChA: Chlorogenic acid; CaA: caffeic acid; Cat: catechin; Que: quercetin; ECa: epicatechin; Rut: rutin; NA; no activity.

that these groups oxidize easily than CoA, GaA, and others PC [29]. On the other hand, comparing the phenolic acid, CoA has no activity on the radical DPPH (Table 1), which suggests that antioxidant capacity depends on the number of hydroxyls [28]. In this way, CaA presented a greater activity than the GaA and ChA. Hydroxycinnamic acids present greater activities than hydroxybenzoic acids. This may be because the CH=CH-COOH group may provide a greater capacity to donate H atoms and stabilize the radical than the −COOH group [30]. CoA did not present inhibition on the DPPH radical, probably due to the fact that the alkene group is adjacent to the carboxylic group (Fig. 1), interferes with the transfer H atoms from the carboxylic group [31]. It should be noted that antioxidant capacity depends on the structural characteristics and physicochemical properties of PC.

In the case of flavonoids, Que presented the highest IC_{50}, followed by ECat and Rut. Their IC_{50} values are in the range of previously reported studies (Table 1). The antioxidant properties of the Que are due to the substitution of hydroxyl 3, 5, 7, 3′, and 4' (Fig. 1); also, presence of catechol in the B ring. The substitution of hydroxyl in positions 3 and 5 of ring C in this flavonoid, confers a greater antioxidant capacity than Cat, since Cat lacks this chemical property. Ruth presented a lesser inhibition than on the radical DPPH. The potential of PC to capture free radicals depends on the number and position of the free −OH groups present in the molecule [2,32,33]. The strength of the hydrogen bridge of hydroxyl 3, 5, 7, 3′, and 4′ also plays an important role in the antioxidant capacity of the PC [34,35].

3.2. Ferric reducing antioxidant power

As can be seen in Table 1, our FRAP values are between the intervals of those previously reported by different authors. Comparing phenolic acids with flavonoids, this table shows that aglicone flavonoids showed higher activity than phenolic acids (Cat>Eca>Que>GaA). According to Morgan [36], the ability of PC to reduce iron is related to the high nucleophilic character of aromatic rings and to chelating groups (substitute groups) within the molecule. In the structure of several flavonoids are three chelatin sites: i) between 5-hydroxy and 4-carbonyl group, ii) between 3-hydroxy and 4-carbonyl group, iii) between 3′, 4′-hydroxy group in B ring [37]. Our results were contradictory to those obtained from Leopoldini et al. [38] that reported that coordination with the catechol group of quercetin is the strongest for iron ion, even greater than catechin. Theoretically, the reduction of iron occurs in the double bond of C2-C3 double bond and the substitution of hydroxyl 3,5 provide hydrogen bonding to the oxo group (present in Que, Fig. 1) by stabilizing phenoxyl radicals through a delocalization of the electorate of phenoxy radicals on the B-ring to the C-ring. Therefore, Que due to its structural characteristics is more favorable in reducing iron. Catechins ((+)-catechin, epicatechin) with a saturated heterocyclic ring lack the essential structural characteristics required for better iron reduction.

Comparing phenolic acids, GaA presented the highest iron ion reducing capacity among phenolic acids. These values may be due to the fact that GaA has three hydroxyl groups that could be favoring the reduction of this ion in acid pH [39,40]. Furthermore, these results can be attributed to the number and position of the hydroxyl groups [42]. Therefore, other analytical studies with GaA and other phenolic acids are necessary to confirm this theory. On the other hand, Sroka & Cisowski [41] observed that hydroxycinnamic acids are better iron reducers than hydroxybenzoic acids at neutral and alkaline pH. The same effect was shown in the study by Andjelkovic [42]. The carboxylic group and the alkene group presented by hydroxycinamic acids (CaA, CoA) have a positive influence on their iron reducing capacity [42,43]. CoA has one hydroxyl group, compared to CaA which has two hydroxyl groups, which makes its ability to reduce metal ions moderate. The number of hydroxyl groups contained in the molecular affects the antioxidant properties [28].

In the case of flavonoids, Cat presented highest chelating capacity, followed by Que>ECat>Rut. This behavior is due to 5-hydroxy-4-keto, 3-hydroxy-4-keto and/or *o*-dihydroxy groups, suggesting that these varieties are important for iron reduction activity [44]. Rut presented a lower iron ion reduction, probably because the conjugation with a carbohydrate reduces its reducing capacity [45].

Table 2
Activity of the binary combination of the phenolic compounds on the inhibition of DPPH radical and chelating of iron by FRAP.

Phenolic compound		DPPH		FRAP	
		Isobologram	CI	%D	Interpretation
GaA	Cat	S	0.27	37.49	S
Cat	Que	S	0.36	5.76	S
Eca	Rut	S	0.47	1.99	S
Cat	ChA	S	0.35	3.14	S
Cat	Rut	S	0.29	0.64	Ad
Cat	ECa	S	0.10	24.33	S
CaA	Eca	S	0.45	−31.01	Ad
GaA	Que	A	3.11	19.25	S
Rut	Que	A	2.25	0.58	Ad
ChA	CaA	A	1.97	−44.21	A
ChA	Rut	A	3.57	−30.06	A
GaA	Rut	A	4.36	0.49	Ad
ChA	Que	A	2.47	−38.62	A
Cat	CoA	A	3.35	−51.30	A
Que	CoA	Ad	0.93	−38.36	A
Eca	CoA	A	2.36	−39.73	A
Que	CaA	A	2.24	−51.52	A
Eca	GaA	Ad	1.05	4.73	S
ChA	CoA	Ad	1.07	−42.24	A
CoA	Rut	Ad	1.01	−2.61	A

GaA: gallic acid; CoA: coumatic acid; ChA: Chlorgenic acid; CaA: caffeic acid; Cat: catechin; Que: quercetin; ECa: epicatechin; Rut: rutin; CI: combination index: CI < 1: synergism, CI = 0 Additive, CI > 1: antagonism; %D: difference: %D positive: synergism; %D negative: antagonism; %D zero: additive; S: synergic; A: antagonic; Ad: additive.

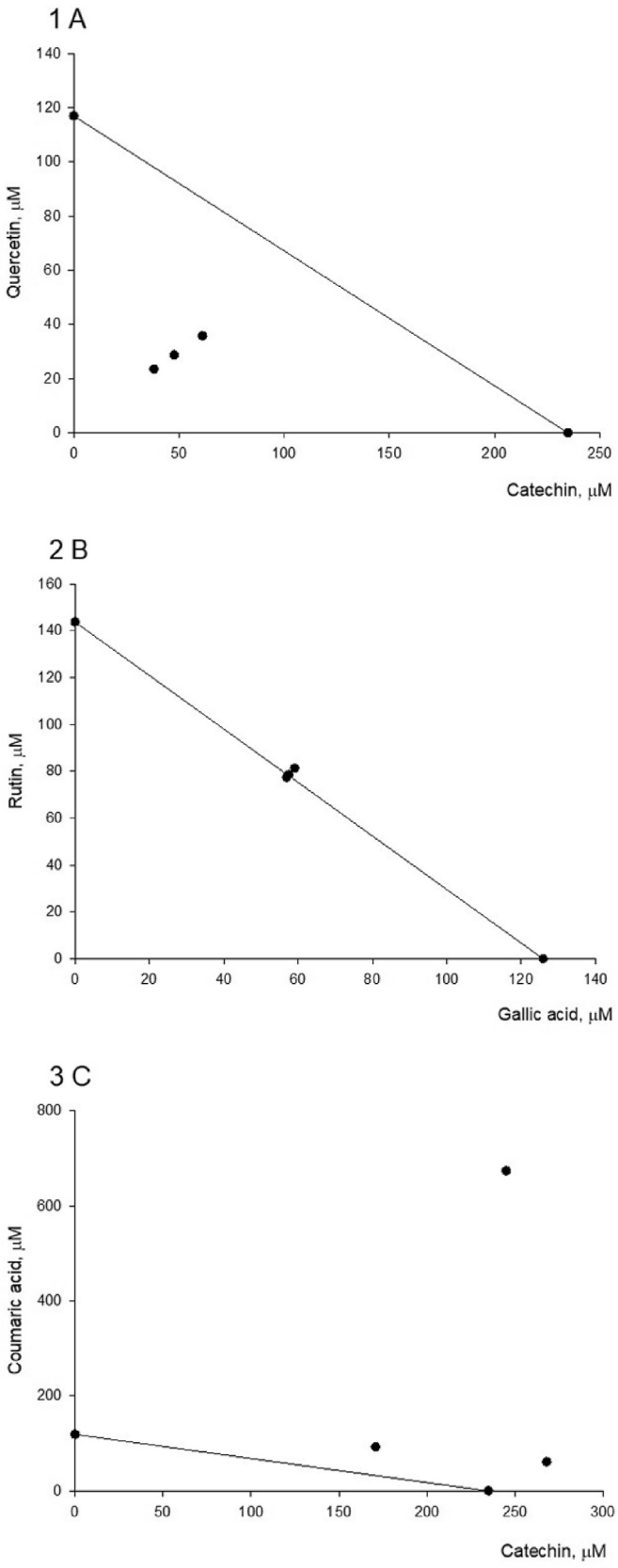

Fig. 2. Comparison of the activity of the binary combination of phenolic compounds on the inhibition of the radical DPPH. 1) Flavonoid vs flavonoid; 2) Phenolic acid vs glucosid flavonoid; 3) Phenolic acid vs flavonoid. A) Synergistic effect; B) Additive effect; C) Antagonistic effect.

3.3. Antioxidant capacity of the binary interactions among phenolic compounds

In order to evaluate the effect of the interactions among PC over their overall antioxidant capacity, twenty PC combinations were evaluated for antioxidant capacity by DPPH and FRAP (Table 2). CoA did not present a DPPH antioxidant capacity, it was used within the combinations, to evaluate the effect of this compound on the activity of the other PC. The effect of the combination of PC by the DPPH was determined by both isololoogram method (graphical) and combination index, and results may be grouped as synergistic, antagonistic or additive. Fig. 2 exemplifies the synergistic (Fig. 2A), additive (Fig. 2B) and antagonistic (Fig. 2C) effects of three combinations of PC analyzed by the isobologram method. It should be noted that there are studies evaluating the mechanisms of PC interactions [46,47], however, there are no studies evaluating the antioxidant capacity of the binary combination of PC by isololoogram method or combination index.

CoA when combined with flavonoids (Cat) (Fig. 2C), exerts an antagonistic effect on the activity of this flavonoid. The antioxidant capacity of flavonoids combined increases with the increase in the degree of hydroxylation, which act synergistically to inhibit the activity of DPPH [4,46,47]. Table 2 shows the response of combined PC to inhibition of DPPH. GaA, when combined with flavonoids, has a synergic effect (Table 2). The synergistic effect was calculated by calculated by using the absorption of the mixtures and calibration curve of $FeSO_4.2H_2O$, and has also been observed in other mixtures of PC binary combinations (GaA:rosmarinic acid, CaA:rosmarinic acid; ChA:rosmarinic acid) that were not considerable in this project [20]. However, Rut (glycosidized flavonoid) when mixed with phenolic acids (CoA, ChA, GaA) present an antagonistic/additive response. It has been demonstrated that favonoids glycosides present lower antioxidant capacity than non-glycosylated, suggesting that glycation impairs the antioxidant capacity of groups responsible for radical elimination and reduction of metal ions [48,49]. The antagonistic effect of the binary interaction Que:Rut on its antioxidant capacity suggests that the attraction force between the glucoside group and the substituent groups weakens the donation of hydrogen ions to inhibit the DPPH radical [44,50]. To confirm the synergism/antagonism effect the CI was determined (Table 2). CI represent the quantitative measurement of combined PC effect, which is more accurate and reliable. Table 2 shows that most of the binary combinations between flavonoids presented a synergistic effect; on the other hand, the antagonistic effect was presented between flavonoids-acid phenolic. Therefore, the antioxidant capacity is influenced by the interactions of the different characteristics of the PC, as well as the interactions between different groups of PC, hydrophobic interactions, hydrogen bonding and ionic interactions and the conformational change of the functional groups (*cis*/*trans*) [7,29,51]. An antagonistic effect was presented in the combination GaA-Que. Considering that GaA is a trihydroxybenzoic, the carboxylic group is directly bound to the benzene ring (Fig. 1) reduces the chelating activity with quercetin (Table 2), because it forms hydrogen bridges with OH group in position 3 (Fig. 1) [39].

On the other hand, the effect of the reducing ion activity of the binary combination of the PC was analyzed, calculating the %D. The chelating activity of flavonoids has been attributed to the presence of 3- or 5-hydroxypyran-4-one and the catechol fraction in the B ring through Fenton type reactions [20,50]. The results of the present research on the synergistic/antagonistic effects between phenolic acids, flavonoids and phenolic acids-flavon (Table 2). Combination of Cat-Rut, CaA-ECa, Rut-Que, and GaA-Rut did not change the antioxidant capacity (additive activity). GaA combined with phenolic acid and flavonoids indicates that the use of this

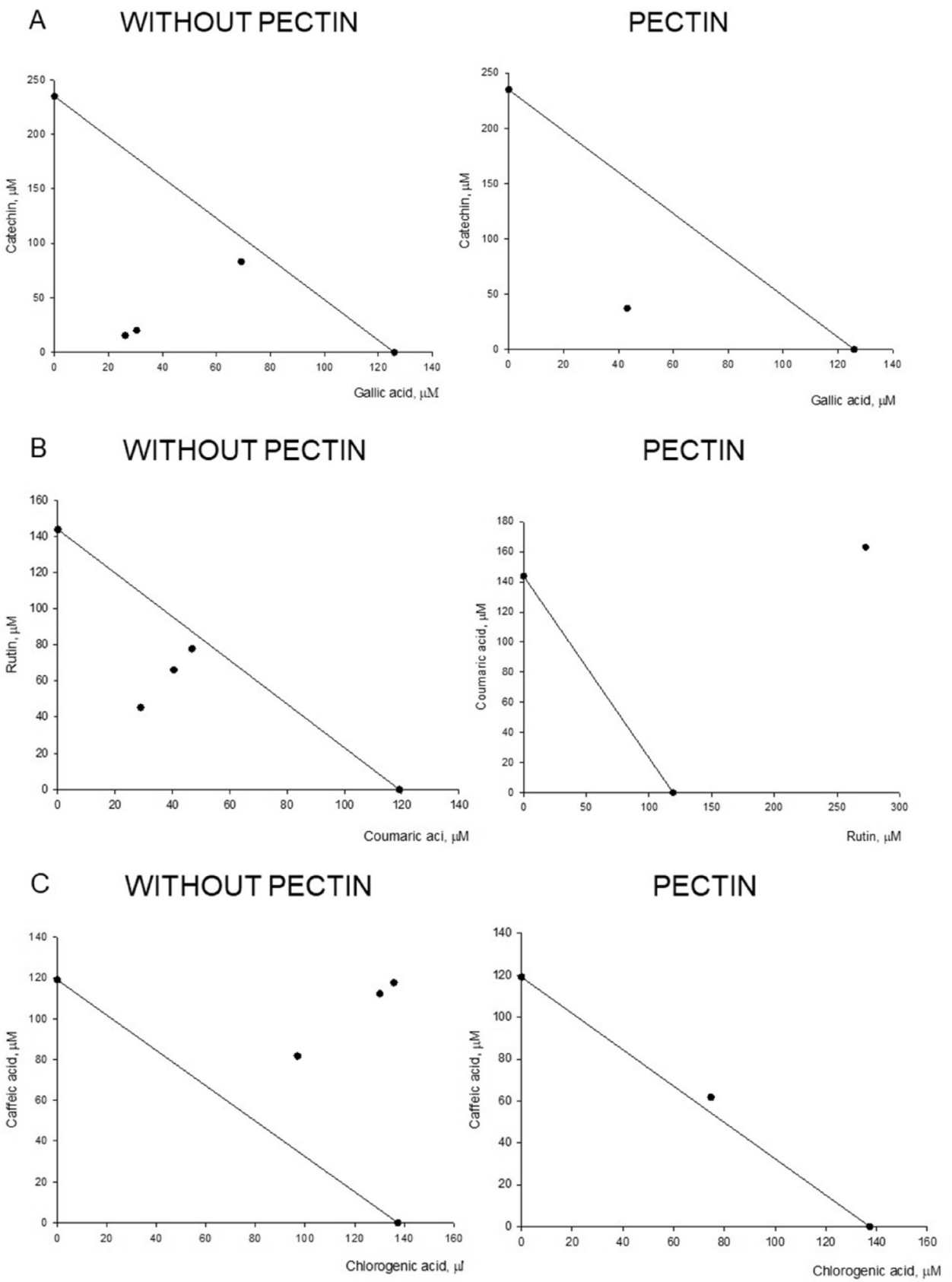

Fig. 3. Comparison of isolobolograms model between the antioxidant capacity of the binary combination of phenolic compounds. A) Phenolic acid vs flavonoid B) Flavonoid vs flavonoid; C) Phenolic acid vs Phenolic acid.

Table 3
Effect of pectin on the binary combination of the phenolic compounds on the inhibition of DPPH radical and chelating of iron by FRAP method.

Compounds			DPPH (IC_{50}, μM)	FRAP (μM)
_	–	Pectin 5%	17.20 ± 0.93	12.13 ± 0.43[a]
_	–	Pectin 10%	NA	10.15 ± 6.68[a]
_	–	Pectin 20%	NA	11.42 ± 0.48[a]
GaA	–	Pectin 5%	39.40 ± 4.00[b]	132.92 ± 2.83[b]
Cat	–	Pectin 5%	23.24 ± 1.94[a]	159.86 ± 2.27[a]
Ecat	–	Pectin 5%	22.77 ± 2.20[a]	99.48 ± 0.14[c]
Rut	–	Pectin 5%	27.30 ± 1.94[a]	42.99 ± 1.57[e]
ChA	–	Pectin 5%	37.45 ± 3.05[b]	45.88 ± 0.76[e]
CaA	–	Pectin 5%	66.46 ± 0.50[c]	63.97 ± 3.64[d]
Que	–	Pectin 5%	15.52 ± 3.80[a]	143.29 ± 8.27[a]

Values are the means of three replicates ± standard deviation (SD) (n = 3). Values with different lower letters are significantly different ($p < 0.05$). GaA: gallic acid; CoA: coumatic acid; ChA: Chlorgenic acid; CaA: caffeic acid; Cat: catechin; Que: quercetin; Eca: epicatechin; Rut: rutin; NA: not activity.

phenolic acid may result in the possibility of maintaining antioxidant capacity in a synergistic manner. When GaA is mixed with other PC the substitution of 3-OH results in an increased angle of torsion and loss of coplanarity, which reduces its antioxidant capacity [51,52]. Some investigations have observed that there is an oxidation in the combination of PC generating an unfavorable effect on the reducing capacity of ions (antagonism/additive) [47,54]. This effect is due to the structural varieties presented by PC, mainly flavonoids, which could be expected to behave as Lewis base [51,54]. In the same way, an antagonistic effect was presented in the combination GaA-Que. Considering that GaA is a trihydroxybenzoic, the carboxylic group is directly bound to the benzene ring (Fig. 1) reduces the chelating activity with Que (Table 2), because it forms hydrogen bridges with OH group in position 3 (Fig. 1) [42].

3.4. *Effect of pectin on the antioxidant capacity on the interaction of phenolic compounds*

Thus, when analyzing the effect of pectin on the antioxidant capacity of the binary combination of PC by the isobologram method. Fig. 3A shows that the synergistic effect prevailed in the combination GaA:Cat, whereas pectin exerts an antagonistic and additive effect on the combination CoA:Rut and ChA:CaA, respectively.

Table 3 shows the effect of pectin on the antioxidant capacity of pure PC. First, inhibition of DPPH radical with pectin was performed at three concentrations, showing a small activity at 5 mg/mL, while at 10 mg/mL and 20 mg/mL no antioxidant activity was observed. Previous studies have shown that DF with different molecular weights may exhibit different antioxidant capacities, particularly in terms of scavenging free radicals [55,56]. Abang-Zaidel [57] observed that the pectin from the shell of the dragon fruit has an antioxidant capacity (81.91%) with an $IC_{50} \leq 0.4$ μg/mL, because PC were found to be present in the structure; however antioxidant capacity decreased to concentrations >0.5 μg/mL due to the rapid competition of hydrogen ion donation to neutralize the DPPH radical. It has been reported that the type of ramification (carboxymethylcellulose, hydroxyethylcellulose, hydroxypropyl methylcellulose and methylcellulose) of pectin influences antioxidant capacity [58].

The binary interaction between pectin and individual PC, increased their antioxidant capacity when measured by both methods (Table 3). This could be because the PCs are complexed within the structural conformation of the DF. Therefore, the type of PC and the content of PC present in different citrus peels influence the antioxidant capacity in the interaction with the DF present in the peels [58,59]. Consequently, the dietary fiber protects the PC from oxidation, favoring a good antioxidant capacity [13,59].

On the other hand, comparing the binary combination of PC with and without pectin showed that there was not much difference in inhibition of DPPH (Tables 1 and 3). This could be due to the dispersed particles, including the average particle size will influence the interaction between PC-DF ratio [55]. Therefore, the reducing capacity of the iron ion was maintained with the presence of DF (Table 3).

Table 4 shows the results obtained from IC_{50} of antioxidant capacity in the presence of pectin. Previous studies have shown that flavonoids (Cat, Que, ECat) have hydrophobic interactions present some contributions to the flavonoid's affinity to cellulose [60]. Likewise, the interactions increase depending on the number of interactions of the OH of flavonoids:pectin [60,61]. It has been observed that the interactions between DF (pectin, cellulose) with phenolic acids are stronger than with flavonoids, due to the carboxylic group [62]. The interactions are more critically dependent

Table 4
Effect of the addition of pectin on the binary combination of phenolic compounds on antioxidant capacity.

Compounds			DPPH (μM)	FRAP (μM)	DPPH			FRAP	
					Isolobogram	CI	Interpretation	%D	Interpretation
GaA	Cat	Pectin 5%	27.71 ± 1.71[b]	143.69 ± 9.08[a]	S	0.50	S	66.11	S
ChA	Rut	Pectin 5%	48.77 ± 1.49[d]	100.16 ± 0.38[c]	A	2.58	A	12.71	S
ChA	CoA	Pectin 5%	13.96 ± 1.62[a]	16.62 ± 0.68[f]	S	0.34	S	0.75	Ad
Cat	Ecat	Pectin 5%	38.39 ± 1.11[c]	139.45 ± 6.42[b]	S	0.91	S	46.23	S
Cat	ChA	Pectin 5%	28.06 ± 0.64[b]	94.63 ± 0.46[c]	S	0.60	S	−54.00	A
ClA	CaA	Pectin 5%	38.57 ± 0.57[c]	37.78 ± 1.73[e]	Ad	1.06	Ad	−65.60	A
Que	CaA	Pectin 5%	26.77 ± 1.79[b]	80.60 ± 0.12[c]	S	0.47	S	0.61	Ad
GaA	Ecat	Pectin 5%	32.72 ± 1.36[c]	89.28 ± 0.27[c]	S	0.81	S	61.58	S
CoA	Rut	Pectin 5%	53.27 ± 1.67[d]	70.94 ± 0.15[d]	A	3.42	A	−12.12	A

Values are the means of three replicates ± standard deviation (SD) (n = 3). Values with different lower letters are significantly different ($p < 0.05$). GaA: gallic acid; CoA: coumatic acid; ChA: Chlorgenic acid; CaA: caffeic acid; Cat: catechin; Que: quercetin; Eca: epicatechin; Rut: rutin; CI: combination index: CI < 1: synergism, CI = 0 Additive, CI > 1: antagonism; %D: difference: %D positive: synergism; %D negative: antagonism; %D zero: additive; S: synergic; A: antagonic; Ad: additive.

on the orientations of the aromatic rings, the type of functional group and the substitution of other components (glucose) linked to the PC aroma ring [35,38].

On the other hand, in the FRAP method it was observed that in the eight combinations of the analyzed PC, the majority presented additive and antagonistic effects (Table 4). This effect may be due to changes in the orientation of the functional groups when interacting with pectin that can significantly change the metal-PC complex. In addition, pectin changes the pH (acid to alkaline) which decreases the chelating activity and the metal-PC complex can occur slowly [58,62]. Determining the %D (Table 4) shows that the effect of the combination of PCs depends on how they are interacting with pectin. Which the iron-reducing activity differs by the interactions between pectin:PC binary combination, which can be noncovalent, hydrophobic, hydrogen bonding, and ionic interactions [60,62]. These interactions could affect the stability, functionality and availability of the OH groups of the PC [63,64]. It should be noted that there are no studies showing whether the interactions between PC-pectin occurs in the internal part, on the surface or adhered to the structure of DF; it is also unknown whether each PC interacts independently with pectin and in different spaces which opens the opportunity to generate studies to deduce the effect of pectin on the interaction of PC.

4. Conclusion

In this study, the effect of binary combinations of PC in the presence and absence of pectin over their antioxidant capacity was evaluated through the isobologram, the combination index analysis and the percentage of difference methods. The flavonoid-flavonoid binary combination showed synergistic effect on the inhibition of DPPH while the combination of phenolic acids and flavonoids showed an antagonistic or additive effect. Pectin interfered with the synergistic effect of PC interactions, modifying electron transfer in antioxidant capacity. The type of hydroxyl groups and/or the substitution of other functional groups and their location influenced the antioxidant capacity of PC individually and binary and trinary combinations.

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