Biofilm Inhibition of Spoilage Bacteria by Argentinean Fruit Juices with Antihypertensive Activity

Claudia V. Vallejo[a], Pedro A. Aredes Fernández[a,b], Marta E. Farías[a,b] and María J. Rodríguez Vaquero[a*]

[a]*Facultad de Bioquímica, Química y Farmacia. Universidad Nacional de Tucumán, Ayacucho 471, (4000) Tucumán, Argentina, UNT-CONICET;* [b]*Centro de Referencia para Lactobacilos (CERELA), Chacabuco 145 (4000) Tucumán, Argentina*

Abstract: Argentinean juices have been studied for their antihypertensive activity, the inhibition of bacteria biofilm formation and the effect on the viability of wine yeast. The influence of phenolic compounds on these activities was evaluated. These studies are the first step for the development of a new type of wine that includes grape must supplement with fruit juices with antihypertensive effect. All juices posses a high antihypertensive activity, higher than 50%. Strawberry juices and eureka lemon showed the highest activity, whereas clarified juices posses the lowest activity. All studied juices produce a high inhibition of bacteria biofilm formation, and the strawberry, orange and mandarin varieties not affect the growth or viability of yeast. Our results permit to conclude that it could be possible the use of these juices in a new type of wine or as a source of new antihypertensive agents for pharmaceutical industry.

Keywords: Antihypertensive activity, Antimicrobial activity, Biofilm inhibition, Fruit juices, Phenolic compounds, Strawberry juice.

INTRODUCTION

Angiotensin I-converting enzyme (ACE) is a peptidyldipeptide hydrolase that plays an important physiological role in both the regulation of blood pressure and cardiovascular function [1]. First, ACE catalyzes the hydrolysis of angiotensin I, an inactive decapeptide, to angiotensin II, a powerful vasoconstrictor and salt-retaining octapeptide. Thus, ACE-inhibition has a hypotensive effect. Secondly, ACE catalyzes the inactivation of the vasodilator bradykinin [2]. Therefore, ACE-inhibitor compounds exert an antihypertensive action.

Phenolic compounds are found in several vegetables and they have a variety of beneficial effects on human health [3, 4, 5]. Wine making is an ancient practice and even today is to improve the aroma and flavor of wine, among others. Keeping these facts in mind, the present study investigates the potential use of Argentinean fruit juices as a source of antihypertensive and selective antimicrobial agents against spoilage bacteria, which could be added to grape must, to make a new type of wine.

There are no reports available that studied the anti hypertensive activity of Argentinean juices, and there are not investigations that considered the possible influence of phenolic compounds from juices on the antihypertensive activity or the effect against biofilm formation of undesirable bacteria for beverages and that not affect the yeast viability, that play an important role on fermentation of grape musts in wines.

The aim of the present study was the study of the antihypertensive activity, the inhibition of bacterial biofilm formation and the viability of wine yeast in Argentinean juices. The relation between these activities and the phenolic compounds concentrations was evaluated. These studies are the first step for the development of a new type of wine that includes grape must be added with fruit juices with antihypertensive effect, in order to increase the beneficial properties for the consumers.

MATERIALS AND METHODS

Fruit Juices

Different fruits were used for this study, three varieties of lemon: *lisboa*, *eureka* and *génova*; One mandarin variety: *murcott*; three orange varieties: *tangerinas*, *valencia late* and *criollas jaffa*; two strawberry varieties: *camarosa* and *albión*. Fresh fruit samples with no apparent physical or microbial damage were collected from farms located in the northwest region of Argentina and purchased by INTA (National Institute of Agricultural technology - 2009), according to season crops at the time of sampling. The fruits were washed with tap water at room temperature and ground in processor to make 1 Liter of juice from each variety. The juices are filtered using a funnel, gauze and cotton, and then centrifuged to separate solids. The supernatant of the juices is split and stored in vials at 4 °C, protected from light. Fruit juices were clarified, to remove phenolic compounds, by the addition of 30 mg/L of activated charcoal. Clarified juices were used as control.

*Address correspondence to this author at the Facultad de Bioquímica, Química y Farmacia. Universidad Nacional de Tucumán, Ayacucho 471, (4000) Tucumán, Argentina, UNT-CONICET; Tel: 54-381-4247752 (ext 7067); Fax: 54-381-4247752; E-mail: mariajo@fbqf.unt.edu.ar

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Phenolic Compounds Concentration

A colorimetric assay based on the Singleton and Rossi technique [6] was carried out to determine the total phenolic concentration. Gallic acid was used to perform a standard curve and results are expressed as milligram per liter gallic acid equivalents (GAE). The phenolic acid and flavonoid concentrations were determined using the method proposed by Zoecklein *et al.* [7], briefly juices were mixed with diluted HCl (1:3) and 8.0 mg/mL formaldehyde solution and incubated 24 h at room temperature in order to precipitate the flavonoid fraction. The phenolic acid contents were determined in the filtrate using the procedure of Singleton and Rossi. The flavonoid content was obtained by the difference between total phenol and phenolic acids content. Results were expressed as mg/L of gallic acid equivalents (GAE).

The phenolic compounds concentrations were confirmed by HPLC analysis, using a Knauer Smartline system chromatograph. The chromatographic conditions were as follows: reprosil-Pur ODS-3 (250 x 4.6 mm), flow rate 1,0 mL/min, injection volume 20µL, detection wavelength 280 nm. Phase A (phosphoric acid 0,1%) and phase B (CH_3CN).

ACE-inhibitory (ACEI) Activity

The antihypertensive activity was determined in the nine juices, clarified juices and in selected pure phenolic compounds normally found in fruits. On the basis of the literature, eight phenolic acids (gallic, protocatechuic, vanillic, p-coumaric, syringic, caffeic, ferulic and chlorogenic acids) and seven flavonoids compounds (rutin, quercetin, quercitrin, myricitin, kaempferol, rhamnetin and ellagic acid) were selected. The phenolic compounds concentration used for the screening of antihypertensive activity was 20 mg/L, concentration normally found in juices.

ACEI activity was determined with slight modifications of the method first described by Cushman and Cheung [8] and later modified by Hernández-Ledesma *et al.* [9]. This technique is based on quantification of hippuric acid formed by the reaction of hippuryl-histidyl-leucine (HHL) with ACE in the presence and absence of an inhibitor. An aliquot of 110 µL of substrate, 10 mM HHL dissolved in a buffer (0.2 M phosphate and 0.3 M NaCl, pH 8.3), and 25 µL of samples (juice, clarified juice or individual phenolic compound). The reaction mixture was incubated at 37 °C for 80 min and then the enzyme reaction was stopped by addition of 110 µL of 1N HCl. The hippuric acid formed was extracted with 1 mL of ethyl acetate, shaken and subsequently centrifuged at 5000 g for 10 min. A 750-µL aliquot of the organic layer was dried at 45 °C in a vacuum chamber (-60 cm Hg) for 60 min. The residue was redissolved in 1 mL of distilled water, and absorbance was measured at 228 nm. A reaction blank was obtained by addition of HCl before ACE enzyme activity. Interference of compounds with absorbance at this wavelength was eliminated with a sample blank. ACEI activity is expressed as follows:

$$\% \text{ of ACEI} = 100[(A\text{-}B) - (C\text{-}D)] / (A\text{-}B) \quad (1)$$

where A represents the absorbance in the presence of ACE, B the absorbance of the reaction blank, C the absorbance in the presence of ACE and inhibitor and D absorbance of the sample blank.

Influence of Juice in Bacterial Biofilm Formation

The bacteria used as test organism *Escherichia coli ATCC 35218* (American Type culture collection), *Escherichia coli* ATCC 25922, *Escherichia coli* ATCC 700, *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 25923.

Briefly, 200 µL of the overnight culture and 50 µL of the selected bacteria was added to the wells of sterile flat bottom 96-well polystyrene microtiter plates and incubated for 24 h at 20 °C for biofilm formation. Then, the non-adherent cells were taking off and the wells washed twice with distilled water in order to remove all non adherent cells, and 200 µL of 0.01% (w/v) Crystal Violet (CV), were added to the wells for 30 min in darkness. The stained biofilms were rinsed with distilled water and extracted with 200 µL of 96% ethanol. The amount of biofilm was quantified by measuring the OD 595 nm of dissolved CV using the microplate reader. A control of biofilm formation of each bacterium was made and un-inoculated medium controls were included, besides, controls carried out with the addition of clarified juice were made. The effect of phenolic compounds of juices was obtained by subtracting the biofilm formation in control media and the effect observed with clarified juices.

Effect of Juice against Wine Yeast

The yeast utilized were *Saccharomyces cerevisiae* mc2 and *Kloeckera apiculata* mc1 isolated from argentinean wine [10]. The yeasts were cultured in YMPG broth and agar medium (contain in g/L: glucose, 20; peptone, 20; yeast extract, 10 and malt extract, 5; pH 5.5).

Minimum Inhibitory Concentration (MIC) and Minimum Microbicide Concentration (MMC)

MIC and MMC were determined in YMPG medium, using a macrobroth dilution method as described by the Clinical and Laboratory Standards Institute [11]. The final concentration of microorganism in each macrobroth dilution tube was approximately 5×10^5 cfu/mL of YMPG. Serial dilutions of juice were used. The MIC was defined as the lowest concentration of phenolic compound that resulted in no visible growth after 24 h of incubation at the optimal temperature of each microorganism. Samples (50 µL) from clear tubes were spread on YMPG agar. The MMC was defined as the lowest concentration of phenolic compounds that resulted in ≥ 99.9% kill of the initial inoculums. The MIC and MMC of clarified juice were also carried out and were used as control. The studies were conducted in triplicate.

Flow Cytometric Analysis of Microbial Viability

Percentage of viable, injured and dead yeast in presence of selected juice was obtained by the use of BD Cell Viability Kit (BD, Biosciences). The kit contains a thiazole orange (TO) solution to stain all cells and propidium iodide (PI) to stain dead cells. Yeasts were grown to exponential phase in optimal conditions and were diluted to approximately 10^6 cfu/mL in staining buffer (physiologic phosphate-buffer saline containing 0.01 % Tween 20). It was added 5.0 µL of each dye solution to 500 µL of cell suspension. The final staining concentrations were 420 nmol/L for TO and 43

μmol/L for PI. The samples were mixed and incubated for at least 5 min at room temperature. Flow cytometric analysis was performed using a BD FACS Calibur flow cytometer (Becton, Dickinson and Co., United States) with an air-cooled argon ion laser (488 nm at 15 mW). This standard instrument was equipped with two light scatter detectors that measure forward (FSC) and side scatter (SSC) and three fluorescence detectors (FL1, 525 nm; FL2, 585nm; FL3, 620 nm). Data were stored as list mode files and analyzed off-line using the FCS Express version 3 software (Beckman Coulter). A combination of FSC and SSC was used to discriminate bacteria from background. TO fluoresced primarily in FL1 and FL2 while PI fluoresced primarily in FL3. Therefore, the best discrimination of live and dead populations was on an FL1 vs. FL3 plot.

Statistical Analysis

All experiments were carried out at least in triplicate. Statistical analysis was performed using MS-Excel software.

RESULTS

Camarosa and *albión* strawberry varieties content the highest concentration of total phenolic compounds between the nine juices studied (Table **1**). The phenolic compounds concentration in *lisboa*, *eureka* and *génova* lemon, *murcott* mandarin and *tangerinas, valencia late* and *criollas Jaffa* oranges were lower than the two strawberry varieties, lemon varieties have the lowest phenolic compounds concentration. In all juices, flavonoid fraction was about 75 % higher than phenolic acids fraction. In clarified juices, the phenolic concentrations were 80% lower, indicating that the clarification process was effective to remove phenolic compounds.

With respect to the pH there were not differences between juices and clarified juices (used as control) and the values were around 2.3 and 5.9 (Table **1**), so any possibly effect of the acidity of the juice was discarded.

The phenolic compounds profile in fruit juices (Table **2**) showed that vainillinic and syringic acids were not detected in juices, whereas ferulic acid and myricetin were present in all of them. In *lisboa* lemon, rutin and quercetin were the main compounds, however in *eureka* and *génova* varieties quercitrin was the main compound, as well as in *murcott* mandarin. Rutin was the major component in *valencia latte* orange and this compound was no detected in the other orange varieties; catechuic was the main compound in *criolla Jaffa* orange. In strawberries juices, p-coumaric acid was the main compound in both varieties. Chlorogenic acid was detected in all juices, but it concentration could not be determine.

Antihypertensive Activity

Fig. (**1**) shows the antihypertensive activity of individual pure phenolic compounds. Results show that ACEI activity of all individual phenolic compounds was higher than 40%. Between phenolic acids, caffeic and gallic acid show the highest ACEI activity, whereas, rutin, quercetin, myricetin and ramnetin present the higher ACEI activity between flavonoids compounds. The lowest activity was shown in presence of syringic acid, ellagic acid and kaempferol (between 38.8 and 40.4%).

The ACEI activity of juices and clarified juices was showed in (Fig. **2**). All juices posses a high antihypertensive activity, higher than 50%. The two varieties of strawberry juices and eureka lemon showed the highest inhibition of ACE activity. The ACEI activity was lower in clarified juices, with values ranged between 2 and 28%.

Influence of Juice in Bacterial Biofilm Formation

Table **3** shows the inhibition on biofilm formation of selected contaminant bacteria in presence of selected juices. The addition of all juice decreased the biofilm formation in all bacteria, and the inhibition was higher than 50%. The high biofilm inhibition was observed in presence of the two

Table 1. Phenolic Concentrations and pH in Argentinean Fruit Juices.

		Juice				Clarified Juice		
	pH	TPC	PA	F	pH	TPC	PA	F
Lisboa lemon	2.3	799.1±40*	165.5±8	633.6±32	2.5	158.2±8	46±3	111.7±6
Eureka lemon	2.3	747.3±37	138.0±7	609.2±31	2.5	156.8±8	45±2	111.7±6
Génova lemon	2.3	519.5±26	108.5±5	411.0±21	2.5	101.6±6	33±2	68.5±4
Murcott mandarin	3.6	752.0±38	153.8±8	598.3±30	3.9	150.0±8	49±3	100.5±5
Tangerina orange	5.9	758.8±38	143.6±7	615.3±31	6.0	152.0±8	40±3	112.1±6
Valencia latte orange	3.3	728.8±37	144.9±7	583.9±29	3.4	190.2±10	59±4	130.9±7
Criolla jaffa orange	3.7	819.5±42	157.3±8	662.3±33	3.9	182.7±9	58±4	125.2±6
Camarosa strawberry	3.6	1257.7±63	203.0±10	1054.7±53	3.8	253.1±13	65±5	187.8±9
Albión strawberry	3.7	1188.4±59	194.3±10	994.1±50	3.9	233.2±12	57±4	176.0±9

*Phenolic compounds concentration (mg GAE/L).

TPC: Total phenolic compounds, PA: phenolic acids and F: flavonoid compounds.

Table 2. Principal Phenolic Compounds Profile in Argentinean Fruit Varieties.

	Retention time (min)	*Lisboa* lemon	*Eureka* lemon	*Génova* lemon	*Murcott* mandarin	*Tangerina* orange	*Valencia latte* orange	*Criolla jaffa* orange	*Camarosa* strawberry	*Albión* strawberry
Gallic acid	4.0	5.0*	nd	nd	nd	0.71	nd	nd	0.20	0.82
Protocatechuic acid	7.4	nd	nd	nd	nd	1.11	0.35	nd	0.10	nd
Vainillinic acid	15.2	nd	nd	nd	nd	nd	nd	nd	nd	nd
Catechuic	16.3	nd	nd	nd	nd	nd	21.56	21.81	16.78	nd
Chlorogenic acid	18.4	nd	nd	nd	nd	nd	nd	3.86	nd	nd
Syringic acid	19.7	nd	nd	nd	nd	nd	nd	nd	nd	nd
p- coumaric acid	29.2	nd	nd	nd	1.10	5.75	3.17	0.28	27.52	15.11
Ferulic acid	31.0	18.25	15.52	1.37	4.21	3.64	3.60	4.66	4.16	3.41
Rutin	32.4	33.59	nd	17.16	nd	nd	46.60	nd	nd	3.00
Quercitrin	33.0	11.12	29.82	22.46	nd	nd	nd	nd	20.03	8.38
Quercetin	33.5	29.45	24.82	nd	72.66	nd	4.24	3.95	7.68	3.30
Myricetin	34.5	2.40	3.06	3.94	18.03	5.68	3.16	3.30	4.42	6.70

Nd: No detected.

*mg/L.

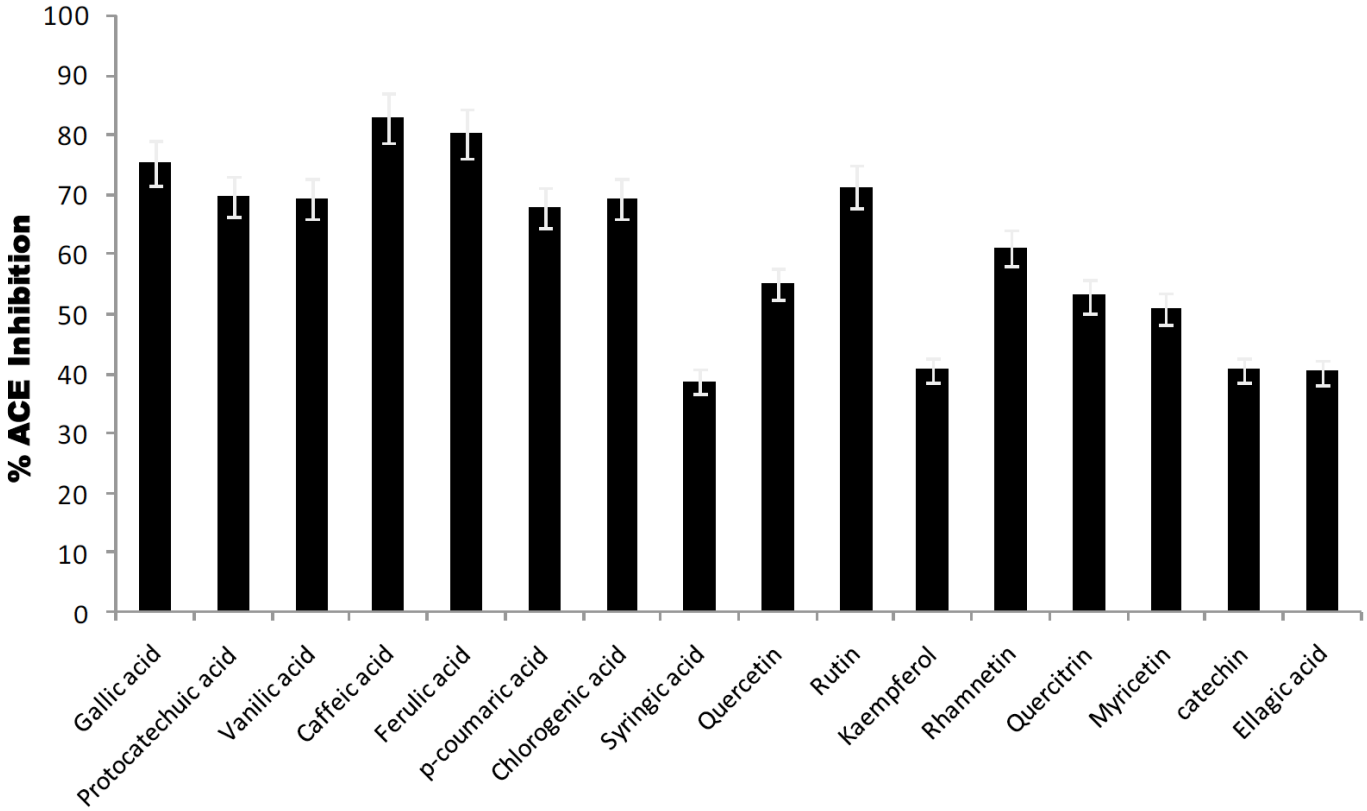

Fig. (1). Angiotensin I-converting enzyme (ACE) inhibition (%) by individual phenolic compounds (20 mg/L). All determination was at least at triplicate.

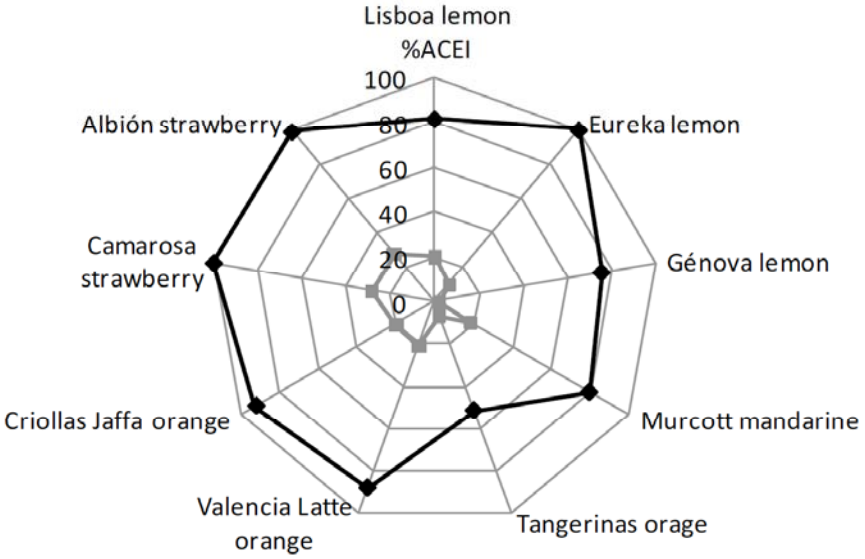

Fig. (2). Angiotensin I-converting enzyme (ACE) inhibition (%) by fruit juices (■) and their clarified juices (■). All determination was at least at triplicate.

varieties of strawberry juices, albión variety reduce 95.5, 70.5, 88.1, 62.9 and 80.9 % the biofilm inhibition, in *E. coli* 25922, *E. coli* 700, *E. coli* 35218, *E. faecalis* and *S. aureus*, respectively. All varieties of lemon juices showed the lowest inhibition with values between 50.1 and 81.6 %. The most sensitive bacteria was *E. coli* 25922 in all cases, and the most resistant bacteria was *E. faecalis*.

Screening of the Antimicrobial Activity of Juices against Wine Yeast

The MIC and MMC of all juices against yeast were presented in Table **4**. The strawberry, orange or mandarin varieties not affect the growth or viability of the two selected yeast. Among the two genera of yeasts evaluated, the growth and viability of *S. cerevisiae* was only inhibited by lemon juices and their clarified juices. Only a concentration of 373.6 mg GAE/L of *eureka* lemon juice was able to produce inhibition and death of *K. apiculata*, but there were not observed inhibition of the growth with clarified juices.

Cytometric Analysis

The percentage of live, damaged or dead cells in the media supplemented with selected fruit juice was evaluated by flow cytometric analysis. Eureka juice was selected according to the CIM and CMM results. Table **5** shows the percentage of live, damaged or dead of eukaryotic cells in presence of eureka lemon juice. No difference was found between e*ureka* lemon juice and its clarified juice; they produce the dead of the 99 % and 94 % of *S. cerevisieae* cells, respectively. These results were in agreement with those obtained with MIC and MMC (Table **4**). In the presence of *eureka* lemon juice, *K. apiculata* showed a major percentage of dead cells (91%) with respect to its clarified juice, where the major proportion of cells were alive (67%).

DISCUSSION

This study demonstrates a high antihypertensive activity of all juices, especially in strawberry varieties that is coincident with their highest phenolic compounds concentration. Clarified juices showed the lowest ACEI activity, which could be related with the loss of phenolic compounds after extraction. Also demonstrate that all individual phenolic compounds studied, normally present in fruit juices posses antihypertensive activity and the great importance of these molecules in the ACEI activity. Ongoing studies on the antihypertensive activity in animal model in order to demonstrate the *in vivo* activity. All studied juices produce an inhibition higher than 50% of the biofilm formation of detrimental bacteria for beverages. Our results are in agreement with those obtained by Puupponen-Pimiä *et al*. [12] demonstrated the antibacterial effect of phenolic compound from berries against intestinal pathogens. Rodríguez Vaquero *et al.* [3, 13, 14] that reported a high antibacterial effect of individual phenolic compounds present in wines. Besides, Rodríguez Vaquero *et al.* [15] reported that phenolic compounds of herb infusions posses a high antioxidant and antibacterial activities. Radovanovic *et al.* [16] demonstrate that southern Serbian red wines with higher amounts of polyphenols and anthocyanins had higher antioxidant and antibacterial properties. There were not differences in pH between juices and clarified juices, so any possibly effect of the acidity on the antihypertensive and antimicrobial activities was discarded.

Keeping in mind that this is the first step for the development was formulating a new type of wine that includes grape must be supplemented with fruit juices with antihypertensive effect. The viability of *Saccharomyces cerevisiae* and *Kloeckera apiculata* that plays an import role in the fermentation of grape must was determined. Concerning antimicrobial activity of fruit juices against yeast, coincident results were found between CIM/CMM and flow cytometric assays. Only lemon juice affected the yeast viability of both studied yeast; therefore, other fruit juices could be considered as a possible source of antihypertensive and antibacterial agents to be included in the beverage production.

Table 3. Inhibition of Bacteria Biofilm Formation by Fruit Juices.

	Percentage of inhibition of bacteria biofilm formation by fruit juices (%)				
	***E. coli* 25922**	***E. coli* 700**	***E. coli* 35218**	***E. faecalis* 29212**	***S. aureus* 25923**
Control	-	-	-	-	-
Lisboa lemon	81.6±2.9	60.0±2.0	80.7±3.0	50.8±2.3	71.6±3.0
Eureka lemon	81.4±2.9	59.5±2.0	79.7±3.0	50.9±2.5	71.9±3.0
Génova lemon	80.3±2.9	58.0±2.0	76.4±2.8	50.1±2.2	71.8±3.2
Murcott mandarin	82.6±2.8	65.2±2.3	82.6±3.3	58.5±2.4	75.0±3.3
Tangerinas orange	83.4±3.0	67.0±2.4	80.9±3.0	58.0±2.8	75.7±3.3
Valencia l. orange	85.2±3.3	66.0±2.5	83.1±3.3	59.5±2.9	79.7±3.6
Criolla j. orange	84.5±3.0	68.0±2.8	85.4±3.5	61.9±2.8	78.9±3.7
Camarosa strawberry	92.2±3.8	70.0±3.0	87.3±4.0	62.5±2.6	79.9±3.7
Albión strawberry	95.5±4.0	70.5±3.1	88.1±4.0	62.9±2.9	80.9±3.9

Table 4. MIC and MMC of Juices against Wine Yeast.

Juice	***S. cerevisiae***				***K. apiculata***			
	Juice		**Clarified juice**		**Juice**		**Clarified juice**	
	CIM	**CMM**	**CIM**	**CMM**	**CIM**	**CMM**	**CIM**	**CMM**
Lisboa lemon	266.4*	266.4	52.7	52.7	Ind	Ind	Ind	Ind
Eureka lemon	249.1	249.1	52.3	78.4	373.6	373.6	Ind	Ind
Génova lemon	173.2	173.2	33.9	50.8	Ind	Ind	Ind	Ind
Murcott mandarin	Ind	Ind	Ind	Ind	Ind	Ind	Ind	Ind
Tangerinas orange	Ind	Ind	Ind	Ind	Ind	Ind	Ind	Ind
Valencia late orange	Ind	Ind	Ind	Ind	Ind	Ind	Ind	Ind
Criolla jaffa orange	Ind	Ind	Ind	Ind	Ind	Ind	Ind	Ind
Camarosa strawberry	Ind	Ind	Ind	Ind	Ind	Ind	Ind	Ind
Albión strawberry	Ind	Ind	Ind	Ind	Ind	Ind	Ind	Ind

* Phenolic compounds concentration (mg GAE/L) found in the juice dilution that produces growth inhibition.

Ind: Inhibition no detected.

CONCLUSIONS

On the basis to the knowledge of these properties will be possible the use of these juices, extensively distributed in our country, to formulate a new type of wine with other characteristics and that increase the beneficial properties for the consumers.

Besides, these juices could be used as new natural additives products for food industry as natural food preservatives or as a source of new antihypertensive agents for pharmaceutical industry.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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