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Nutraceutical properties of *Citrus clementina* juices

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Abstract

The consumption of fruits and vegetables has been inversely associated with morbidity and mortality from degenerative diseases. *Citrus* spp. are the most important commercial fruit crops grown in all continents of the world. It includes different types of fruits and products, important source of bioactive compounds including antioxidants such as ascorbic acid, flavonoids, phenolic compounds and pectins that are important for the human nutrition. It is well known that plant-derived polyphenols have remarkable antioxidant and free radical scavenging activities resulting in multiple beneficial effects in the human health. The antimicrobial abilities are an interesting field for applications within pharmaceutical and food supplement industries. The aim of this study was to investigate and compare the antioxidant and the antimicrobial activities of the juices of 15 clementine cultivars. Thirteen cultivars of Clementine mandarins and two hybrids and Mandalate obtained were investigated. Three assays were used to measure the antioxidant, radical scavenging and antiperoxidative activities of juice extracts: FRAP (Ferric Reducing Antioxidant Power), ABTS radical and Beta-Carotene bleaching (BCB) assays. The values of antioxidant capacity in each data set were transformed into a standard scores so the data can be compared. The mean of all standard scores determines the relative antioxidant capacity index (RACI). The antimicrobial activity and the minimal inhibitory concentration were evaluated against selected bacterial strains by using the agar well diffusion assay. A total of thirty-two gram-negative and gram-positive bacteria were employed as screening microorganisms for this study. Antimicrobial effect and action spectrum of each juice were ALSO evaluated. The study thus revealed that CAF cultivar had the highest antioxidant activity; a considerable value was observed for FED and RA89 cultivars. Instead ORO cultivar showed the lowest RACI. The results showed that no antimicrobial activity was demonstrated by the different citrus extracts against the tested bacteria, but the MAN extract that had an antimicrobial effect on some gram-negative and gram-positive bacteria. Our results confirmed the antioxidant activity of *Citrus clementina* juices, underling wide differences between them, but they also demonstrated that none but MAN juice possess a relevant antimicrobial activity, especially against pathogen bacteria tested. This findings can be helpful in selecting possible extracts to be used as food supplements for antioxidant and/or antimicrobial purposes.

Introduction

It has been demonstrated as cell exposition to high level of reactive oxygen species (ROS) is one of the main cause cardiovascular diseases and some types of cancer [1]. In fact ROS, (e.g., superoxide and singlet oxygen radicals) and nitrogen radicals (e.g., peroxynitrite and nitrogen dioxide radical species generated in vivo) are known to alter cellular structure and function. Several authors demonstrated that diets high in fruits and vegetables are protective against these diseases [2]. In fact fruits and vegetables are differently rich in antioxidant bioactive compounds. This compounds belong to different classes of secondary metabolites that has been shown to possess this activity [3]. However, recent studies have demonstrated that this fruits also contain other bioactive compounds including flavonoids, coumarins, carotenoids, and limonoids with potential health promoting properties. Accumulative evidences suggest antioxidant activities of flavonoids from a variety of plant sources [4-9]. In particular *Citrus* spp. fruits are also rich sources of vitamin C (ascorbic acid), an essential nutrient with well-described antioxidant properties [10-12].

Recently it was reported the influence of variety and species on the content of the main antioxidant constituents (flavonoids, carotenoids, vitamin C, etc.) present in *Citrus* spp. [13-15] and this difference reflect directly the properties of the extracts. It was also reported the chemical composition relative to the content of antioxidant constituents narirutin (NAR), HESP and total vitamin C, as essential nutrients with well-described antioxidant properties present in *Citrus* spp., of the most commercial and productive varieties cultivated in Spain and in Italy [9, 16]. Commonly available citrus fruits, mainly oranges and lemons, have been analyzed thoroughly in order to develop a citrus food composition table [17].

Recently Milella et al. [9] investigated mandarin juice bioactive compounds, total phenolic content and their antioxidant activity only using DPPH test, because mandarins are prized for the delicious flavor of their fresh fruit, but relatively little information is available on the quality and the biological activity of their secondary metabolites when compared to other citrus, such as orange or lemon [18].

It was also demonstrated as citrus peels are rich in nutrients and contain many phytochemicals. In fact they were investigated for their antimicrobial activity by Kumar et al. [19]. Since there is an increase in the number of antibiotic resistance pathogens, there is always a search of an alternative drug that is regarded as safe and possess antibacterial activity.

So far, studies on the comparison of different antioxidant tests and the antimicrobial activity of mandarin extracts haven't been reported yet. If demonstrated in this fruit juices extracts, these can be also used in food supplement industry for their nutraceutical properties, for preventing oxidative stress and/or corroborate natural defenses against bacterial grown.

In this study the antioxidant and antimicrobial activity of 13 cultivars of Clementine mandarins and 2 hybrids were investigated for their potential application as food supplement.

Materials and Methods

Chemicals

Sodium acetate trihydrate, 2, 4, 6-tripyridyl-s-triazine (TPTZ), iron (III) chloride ($FeCl_3$*$6H_2O$), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), potassium persulphate, beta-carotene, linoleic acid, Tween 20, Trolox, ascorbic acid, and all media for bacteria grown were purchased from Sigma-Aldrich (Milano-Italy). Chloroform, hydrochloric acid and glacial acetic acid were purchased from Carlo Erba (Milano-Italy).

Samples collection

Thirteen cultivars of Clementine mandarins (*Citrus clementina* Hort. Ex. Tan) [Caffin (CAF);

Corsica 2 (COR); Esbal (ESB); Fedele (FED); Isa (ISA); Oronules (ORO); Precoce di Massafra (MAS); Ragheb (RAG); Rubino (RUB); Spinoso (SPI); SRA 63 (RA 63); SRA 89 (RA 89); SRA 92 (RA 92)] and two hybrids [Etna (ETNA) (*Citrus unshiu* x *Citrus clementina*) and Mandalate (MAN) obtained from Fortune mandarin (*Citrus clementina* x *Citrus tangerina* Hort. ex Tan.) x Avana Mandarin (*Citrus deliciosa* Tan.)] were investigated. The fruits were grown in Southern Italy (Vivaio Di Natale, Metaponto, Basilicata region) on the same conditions (soil, irrigation and illumination). Sampling of the fruits was carried out in December 2009. The Clementine mandarins were squeezed and 3 mL of juice was lyophilized and extracted twice with 10 mL of methanol. The supernatants were combined and concentrated to dryness at room temperature under a stream of nitrogen. The solid residue was then dissolved in 3 mL of methanol, and then solid-phase extraction (SPE) was used to remove sugars. Extract were stored at -20 °C.

In-vitro antioxidant activity

Ferric reducing antioxidant power (FRAP) assay.

The FRAP assay was carried out as described by Benzie and Strain 1999 [20]. The FRAP reagent was made fresh before each experiment. The FRAP reagent was prepared by mixing 300 mM acetate buffer in distilled water pH 3.6, 20 mM $FeCl_3$*$6H_2O$ in distilled water and 10 mM 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ) in 40 mM HCl in a proportion of 10:1:1. For each sample 150 µL of appropriately diluted sample and 1350 µL of FRAP reagent was added and incubated at 37 °C for 40 min in the dark. In the case of the blank 150 µL of methanol was added to 1350 µL of FRAP reagent. The absorbance of the resulting solution was measured at 593 nm by spectrophotometer. Trolox (6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic Acid) was used as a reference antioxidant standard. FRAP values were expressed as mg Trolox (mg Tr)/100 ml of juice. Each sample was performed in triplicate.

ABTS assay.

The ABTS assay [21] was employed to determine the total antioxidant activity of fruit extracts. Two stock solutions, 7.4 mM ABTS solution and 2.6 mM potassium persulfate solution, were mixed in equal quantity and allowed to react for 12-16 h at room temperature in the dark. The working solution was diluted by mixing 1 mL ABTS solution with 60 mL methanol. The Clementine juice (75 µl) was added at 1425 µl of the working solution for 3 h in the dark. The absorbance was measured at 734 nm by using the spectrometer. Results were expressed as mg Tr/100 ml of juice. Fresh working solution was prepared for each assay and each sample was carried out in triplicate.

Beta-ne bleaching (BCB) assay.

The antioxidant activity was evaluated by the β-carotene-linoleic acid bleaching method [22]. β-carotene solution (0.2 mg of β-carotene dissolved in 0.2 mL of chloroform), linoleic acid (20 mg) and Tween 20 (200 mg) were mixed. Chloroform was removed by using rotary evaporator at 40°C. Distilled water (50 mL) was added with oxygen. Four milliliters of the emulsion were transferred into several tubes containing 0.2 mL of extracts or ethanol as control. BHT was used as positive control. The tubes were placed at 50°C for 3 h. The absorbance was measured at 470 nm by using spectrometer. Each sample was carried out in triplicate. Results were expressed as percentage (%) of β-carotene bleaching inhibition [23].

Antimicrobial activity assay

Bacterial strains

The different citrus extracts were tested against a panel of bacterial strains shown in table 1.

A total of thirty-two strains of the culture collection of the Dipartimento di Biologia, Difesa e Biotecnologie Agro-Forestali, Università degli Studi della Basilicata, Potenza, Italy, were employed as screening microorganisms for this study. All strains were maintained as freeze-dried stocks in reconstituted (11% w/v) skim milk, containing 0.1% w/v

ascorbic acid and routinely cultivated in optimal growth conditions (table 1). These bacteria were chosen in order to represent a complete collection of gram positive and gram negative, pathogenic and non pathogenic once.

Agar well diffusion assay and Minimum Inhibitory Concentration

Antimicrobial activities of all tested extracts were determined by standard agar well diffusion assay [24]. For each strain, a subculture in a specific broth was obtained from the active stock culture by 1% (v/v) inoculum and incubated overnight at the corresponding culture temperature. 200 µl of each subculture was used to inoculate the agar media (to achieve a final concentration of 10^6 CFU/ml) and distributed into Petri plates. 50 µl of each extract was poured into wells (6 mm diameter) bored in the agar plates and then the plates were incubated at optimal growth conditions for each strain. Organic solvent was used as negative control while antibiotic was used as positive control. The experiment was performed in triplicate and the antimicrobial activity of each extract was expressed in terms of zone of inhibition diameter mean (in mm) produced by the respective extract after 24 h of incubation. A inhibition zone <9 mm indicated a low antimicrobial activity; 10< zone of inhibition <14 mm, a middle antimicrobial activity; a zone of inhibition >15 mm, an high antimicrobial activity.

Extracts producing an inhibition zone were screened to determine minimum inhibitory concentrations (MICs) in order to evaluate the antimicrobial effectiveness of each extract against different bacterial strains by the agar well diffusion method [24]. Each specific medium inoculated with the strain subculture was distributed into Petri plates and different concentrations of extracts, ranging from 1 µg/ml to 100 µg/ml, were poured into wells bored in the agar plates and the plates were incubated for 24 h. After incubation, the MIC was calculated as the lowest concentration of the extract inhibiting the growth of bacterial strains. The MIC values were done in triplicate.

Results and discussion

The beneficial effects derived from phenolic compounds have been attributed to their antioxidant activity [25]. The antioxidant activity of an antioxidant compound is related to various mechanisms, among which are prevention of a chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity [26]. A higher absorbance indicates a higher ferric reducing power. For the determination of the reductive ability, we investigated the Fe^{3+}/ Fe^{2+} transformation in presence of the extracts by using FRAP assay. Results showed that FED and CAF possess the highest activity 195.30 and 193.16 mg Tr/100 mL of juice respectively, while MAN demonstrated to be more than twice less active (86.96 mg Tr/100 mL). The mean FRAP values was 138.63 mg Tr/100 mL of juice (table 2). The reducing power of the extracts increases with the increasing concentration. Single assay cannot determine completely the antioxidant activity, different approaches are needed to understand the different biological activity of complex mixture of secondary metabolites [27]. In fact the antioxidant activity of the extracts was tested by using others two complementary systems, ABTS and β-carotene bleaching assays. Free radical scavenging activity of the extracts was assessed by the ABTS assay. The method is based on the ability of antioxidant compounds to quench the long-lived ABTS radical cation (ABTS+) activated by potassium persulfate. CAF, FED, RAG and RA89 extracts exhibited the highest scavenging activity from 23.77 to 25.52 mg Tr/100 mL of juice respectively, while MAN extract has only 7.68 mg Tr/100 mL. The mean ABTS values was 19.58 mg Tr/100 mL juice (table 2).

In the β-carotene bleaching assay, the antioxidant transfers hydrogen atoms to the peroxyl radicals obtained from the oxidation of linoleic acid and converts them to hydroperoxides leaving β-carotene molecules intact. Thus, the degradation

rate of β-carotene depends on the anti-peroxidative activity of the extracts. Unlike the previous methods, MAN extract demonstrated the highest antioxidant activity percentage (%AA), while CAF had the lowest value. The mean value obtained by using β-carotene bleaching assay was 23.40 (%AA). Data are shown in table 2.

Similar information about Clementine juices radical scavenging activity were previously obtained. DPPH assay was used by Milella et al [9] and it is based on the reduction of stable DPPH nitrogen radicals in presence of antioxidants. Although minimal differences were observed between the ABTS and the DPPH values, the methods were equally good at measuring radical scavenging activity and gave similar results with the exception of CAF and MAN extracts [9, 28]. The differences are also congruent with findings obtained by Tabart et al., they found correlation between the two methods but with differences due to the slight molecular diversity [29].

In this paper we use a new concept, relative antioxidant capacity index (RACI) developed by integrating antioxidant capacity data determined by different methods. We used the data of total polyphenols [9] content (TPH) to obtain the RACI index, results are shown in the figure 1. This concept not only allows the comparison of food antioxidant capacity derived from different chemical methods, but also provides a more comprehensive comparison [30]. The extracts analyzed certainly possess wide differences. They certainly have different amounts of phenolic compounds and probably contain different secondary metabolites in their composition. This fact explain why the extracts demonstrated different antioxidant capacities especially measured with different assays.

According to all used tests, the highest antioxidant capacity was observed in CAF and FED extracts, expressed as RACI value, while ORO extract has the lowest.

The antimicrobial activity and the MIC were evaluated against selected bacterial strains of significant importance for human health, pathogenic or non-pathogenic by using the agar well diffusion assay. A total of thirty-two gram-negative and gram-positive bacteria were employed as screening microorganisms to determine the antimicrobial effect, the action spectrum and the antimicrobial effectiveness of each extract. Results showed that all citrus extracts demonstrated no antimicrobial activity against the tested bacteria, but MAN extract. It provided an antimicrobial effect against a wide range of bacteria (table 2). All Gram-negative bacteria were sensitive to the MAN extract with a middle-low antimicrobial activity (inhibition zone ranging from 9.77 to 11.32 mm); while the antimicrobial activities observed varied with the type of tested Gram-positive bacterium. As pathogenic bacteria tested, the MAN extract was more effective against *Listeria monocytogenes* with 17.16 mm diameter of inhibition zone, while *Salmonella serovar* and *Escherichia coli* showed with a low sensitivity to the same extract. MAN extract was found most effective against *Enterococcus* spp. strains with an high antimicrobial activity, except for *Enterococcus gallinarum*, while the inhibition of *Weissella* spp. strains was different according to species tested. For *Weissella hellenica* and *Weissella paramesenteroides* no inhibition zone was observed, while the other strains were sensitive to the MAN extract with a middle-low antimicrobial activity, ranging from 12.78 to 8.83 mm. Moreover, among *Staphylococcus* spp. strains, the MAN extract had a middle antimicrobial effect on a *Staphylococcus xylosus* and a *Staphylococcus equorum* strain, while, none of the extracts exhibited the antimicrobial effect on *Lactobacillus* strains tested, that is a result that possibly indicate, in case of oral administration, the respect of the intestinal flora.

The active extract thus obtained was subjected to determine MIC by the agar well diffusion method against respective susceptible bacterial species (table 3). The results indicated that Gram-negative bacterial species were more sensitive as compared to Gram-positives. In particular *Pseudomonas fragi* and *Pseudomonas proteamaculans* were inhibited at a very low concentration (<1 µg/ml). The MAN extract showed a MIC of 10 µg/ml for two

Staphylococcus spp. strains, resulted sensitive to this extract, while the strains belonging to *Enterococcus* and *Weissella* species required an inhibitory concentration ranging from 80 to 100 µg/ml and also >100 µg/ml. While among pathogenic bacterial species, *Escherichia coli* and *Salmonella serovar* were the most sensitive with a MIC of 5 µg/ml, on the contrary *Listeria monocytogenes* required an inhibitory concentration >100 µg/ml.

Analyzing the different microbiological and antioxidant (measured with BCB assay) of MAN extract, it is probable that the antioxidant components in the MAN juice extract could be constituted by antioxidants more active in lipophilic media. In fact according to the 'polar paradox theory', a protective membrane could be formed around the oil droplets non polar lipophilic antioxidants (e.g. BHA or BHT). Instead the polar hydrophilic antioxidant (e.g. ascorbic acid that was demonstrated to be the lowest among this extracts [9]) will be markedly diluted by moving to the water phase, as it reduces the protection of linoleic acid. Furthermore, fruits belonging to MAN hybrid cultivar showed the lowest antioxidant activity as well as vitamin C and TPH content, while titrable acidity and naringin levels were the highest [9]. Thus a more active antioxidant in lipophilic systems shows greater antioxidant activity in emulsion condition such as the β-carotene-linoleic acid emulsion system, whereas a hydrophilic antioxidant will be more effective in aqueous system (ABTS and FRAP methods) [23]. MAN extract confirmed to be the most active in BCB assay and the only one able to inhibit bacterial grown that means the presence of different molecules or different relative composition of this extract in comparison with the others.

In conclusion, the results of this study highlighted that health-promoting compounds of juices coming from the most important Italian Clementine cultivar. As far as the antioxidant properties of the various juices tested, of interest is the fact that only PCA analysis performed previously by Milella et al [9] demonstrated as MAN was definitely far from the rest of the other cultivar. This correspond to the different chemical composition of its juices. All this results are in agreement with ours. The results showed that the different citrus juice extracts demonstrated no antimicrobial activity but MAN extract. It showed an antimicrobial effect on some gram-negative and gram-positive bacteria. The MAN extract inhibited the growth of all food spoilage bacteria, with a middle-low activity of inhibition against *Serratia proteamaculans* and *Escherichia coli* after 24 h of incubation, while *Hafnia alvei*, *Pseudomonas fragi* and *Brochothrix thermosphacta* were sensitive to the MAN extract only after 48 h of incubation. Moreover, among *Staphylococcus* spp. strains, the MAN extract had a middle antimicrobial effect on a *Staphylococcus xylosus* and a *Staphylococcus equorum* strain, while, none of the extracts exhibited the antimicrobial effect on *Lactobacillus sakei* strains tested. Our results confirmed the antioxidant activity of Citrus clementina juices, underling wide differences between them, but they also demonstrated that none but MAN juice possess a relevant antimicrobial activities.

We can conclude that RA92, FED and CAF juice extracts showed the highest RACI index. This result suggest as this cultivars can be suitable to be used as antioxidant supplements. Conversely, MAN juice extract is certainly the most active against bacteria and also its cell membrane prevention is the highest for this reasons its applications must be focused on its ability in protecting membrane from oxidative stress and prevent bacterial grow with respect of the intestinal flora.

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Strain	Bacterial species	Growth conditions	
		Temperature	Medium
9P	*Carnobacterium maltaromaticum*	20°C	Tryptone Soya Yeast Extract Medium
H02	*Carnobacterium divergens*	20°C	Tryptone Soya Yeast Extract Medium
6P2	*Pseudomonas fragi*	20°C	Tryptone Soya Yeast Extract Medium
53M	*Hafnia alvei*	30°C	Tryptone Soya Yeast Extract Medium
42M	*Pseudomonas proteamaculans*	30°C	Tryptone Soya Yeast Extract Medium
7R1	*Brochothrix thermosphacta*	20°C	Tryptone Soya Yeast Extract Medium
32	*Escherichia coli*	37°C	Tryptone Soya Yeast Extract Medium
LMG6399	*Enterococcus hirae*	37°C	M17 Medium
ATCC14434	*Enterococcus faecium*	37°C	M17 Medium
ATCC14433	*Enterococcus faecalis*	37°C	M17 Medium
ATCC14436	*Enterococcus casseliflavus*	37°C	M17 Medium
ATCC11576	*Enterococcus durans*	37°C	M17 Medium
LMG13129	*Enterococcus gallinarum*	37°C	M17 Medium
DSM 20410	*Weissella viridescens*	30°C	MRS Medium
DSM 20196	*Weissella confusa*	30°C	MRS Medium
DSM 7378	*Weissella hellenica*	30°C	MRS Medium
DSM 15878	*Weissella cibaria*	30°C	MRS Medium
DSM20288	*Weissella paramesenteroides*	30°C	MRS Medium
DSM20014	*Weissella minore*	30°C	MRS Medium
DBPZ0062	*Lactobacillus sakei*	30°C	MRS Medium
DBPZ0416	*Lactobacillus sakei*	30°C	MRS Medium
DBPZ0329	*Lactobacillus sakei*	30°C	MRS Medium
DBPZ0338	*Lactobacillus sakei*	30°C	MRS Medium
DBPZ0098	*Lactobacillus sakei*	30°C	MRS Medium
DBPZ0224	*Staphylococcus xylosus*	30°C	Tryptone Soya Yeast Extract Medium
DBPZ0248	*Staphylococcus equorum*	30°C	Tryptone Soya Yeast Extract Medium
DBPZ0044	*Staphylococcus equorum*	30°C	Tryptone Soya Yeast Extract Medium
DBPZ0251	*Staphylococcus succinus*	30°C	Tryptone Soya Yeast Extract Medium
DBPZ0241	*Staphylococcus equorum*	30°C	Tryptone Soya Yeast Extract Medium
BL/26	*Listeria innocua*	30°C	Tryptone Soya Yeast Extract Medium
DBPZ001	*Listeria monocytogenes*	30°C	Tryptone Soya Yeast Extract Medium
DBPZ002	*Salmonella serovar*	30°C	Tryptone Soya Yeast Extract Medium

Table 1. Bacterial strains and growth conditions used for antimicrobial activity assay

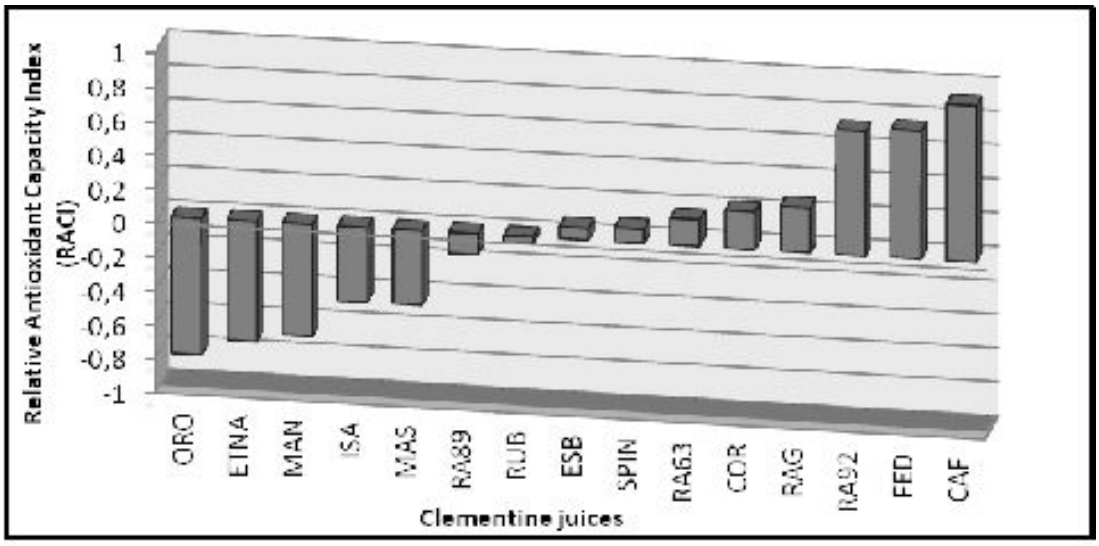

Figure 1. RACI for different Clementine juices

SAMPLE	FRAP (mg Tr/100 ml)	ABTS (mg Tr/100 ml)	BCB (% AA)
CAF	193.15 ± 5.55	23.58 ± 1.92	16.74 ± 0.61
COR	134.89 ± 3.43	16.71 ± 1.53	23.76 ± 1.31
ESB	135.80 ± 3.43	18.19 ± 1.77	27.21 ± 1.85
ETNA	106.05 ± 2.36	12.65 ± 0.75	24.52 ± 1.47
FED	195.30 ± 7.66	22.78 ± 1.74	20.78 ± 1.04
ISA	116.70 ± 5.54	26.85 ± 0.46	23.51 ± 1.57
MAN	86.96 ± 3.37	7.68 ± 0.20	38.16 ± 1.16
MAS	111.55 ± 5.97	19.70 ± 1.91	18.60 ± 1.38
ORO	103.16 ± 4.86	18.94 ± 1.44	17.33 ± 1.80
RA63	140.25 ± 3.32	20.98 ± 0.57	27.54 ± 1.19
RA89	176.07 ± 4.46	25.52 ± 1.51	17.72 ± 1.33
RA92	149.31 ± 3.41	22.34 ± 1.05	24.38 ± 1.40
RAG	124.87 ± 2.68	24.04 ± 1.08	26.66 ± 0.97
RUB	127.09 ± 3.40	21.92 ± 1.11	24.09 ± 1.53
SPIN	178.25 ± 5.39	21.83 ± 1.21	20.02 ± 1.34

Table 2. Antioxidant activity of Clementine juice extracts using FRAP, ABTS and BCB assays

Bacteria	Zone of inhibition (mm diameter)[a]	Concentration of extract (µg/ml)[b]										MIC (µg/ml)[c]
	MAN citrus extract	1	5	10	20	40	60	70	80	90	100	
Gram-positive bacteria												
Carnobacterium maltaromaticum	10.34 ± 1.23	-	-	-	-	-	-	-	-	-	+	100 ± 1.76
Carnobacterium divergens	15.42 ± 0.99	-	-	-	-	-	-	-	-	+	+	90 ± 1.33
Brochothrix thermosphacta	10.58 ± 1.07	-	-	-	-	-	-	-	-	-	-	>100
Enterococcus hirae	12.67 ± 0.93	-	-	-	-	-	-	-	-	-	-	>100
Enterococcus faecium	15.16 ± 1.10	-	-	-	-	-	-	-	-	-	+	100 ± 0.98
Enterococcus faecalis	16.08 ± 0.89	-	-	-	-	-	-	-	+	+	+	80 ± 0.88
Enterococcus casseliflavus	11.46 ± 0.23	-	-	-	-	-	-	-	-	-	-	>100
Enterococcus durans	18.02 ± 1.03	-	-	-	-	-	-	-	-	+	+	90 ± 1.11
Enterococcus gallinarum	8.96 ± 0.35	-	-	-	-	-	-	-	-	-	-	>100
Weissella viridescens	12.78 ± 0.66	-	-	-	-	-	-	-	-	-	+	100 ± 1.08
Weissella confusa	8.83 ± 0.79	-	-	-	-	-	-	-	-	-	-	>100
Weissella hellenica	NI											
Weissella cibaria	10.87 ± 0.28	-	-	-	-	-	-	-	-	-	-	>100
Weissella paramesenteroides	NI											
Weissella minore	9.88 ± 0.77	-	-	-	-	-	-	-	-	-	-	>100
Lactobacillus sakei	NI											
Lactobacillus sakei	NI											
Lactobacillus sakei	NI											
Lactobacillus sakei	NI											
Lactobacillus sakei	NI											
Staphylococcus xylosus	12.47 ± 0.77	-	-	+	+	+	+	+	+	+	+	10 ± 0.58
Staphylococcus equorum	NI											
Staphylococcus equorum	NI											
Staphylococcus succinus	NI											
Staphylococcus equorum	10.82 ± 0.53	-	-	+	+	+	+	+	+	+	+	10 ± 0.12
Listeria innocua	NI											
Listeria monocytogenes	17.16 ± 1.22	-	-	-	-	-	-	-	-	-	-	>100
Gram-negative bacteria												
Pseudomonas fragi	10.11 ± 0.12	+	+	+	+	+	+	+	+	+	+	<1
Hafnia alvei	10.86 ± 0.23	-	-	-	-	-	-	-	-	-	-	>100
Pseudomonas proteamaculans	11.32 ± 0.28	+	+	+	+	+	+	+	+	+	+	<1
Escherichia coli	9.77 ± 1.03	-	+	+	+	+	+	+	+	+	+	5 ± 0.23
Salmonella serovar	9.93 ± 0.88	-	+	+	+	+	+	+	+	+	+	5 ± 0.07

Table 3. Antimicrobial activity and MIC of MAN citrus extract

[a] Zone of inhibition <9 mm: low antimicrobial activity; 10< zone of inhibition<14 mm: middle antimicrobial activity; zone of inhibition >15 mm: high antimicrobial activity.

NI: no inhibition zone was observed. Values of the observed zone of inhibition (in mm diameter) including the diameter of well (6 mm) after 24 h of incubation against different bacterial species when subjected to different extracts in agar well diffusion assay. Assay was performed in triplicate and results are the mean of three values ± Standard Deviation.

[b] Different concentrations of man citrus extract evaluated by the agar well diffusion method as described by Tremonte et al. (2007).

All values are expressed in µg/ml; (–) represents "no inhibition observed"; (+) represents "inhibition observed".

[c] Values are presented as mean ± standard deviation.

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