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CITRUS FLAVONOIDS AS BIOACTIVE COMPOUNDS: ROLE,
BIOAVAILABILITY, SOCIO-ECONOMIC IMPACT AND
BIOTECHNOLOGICAL APPROACH FOR THEIR MODIFICATION

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Abstract

Numerous epidemiological and experimental studies indicate that a variety of food components have biological activity that exhibit a potential for modulating human metabolism in a manner favourable for the prevention of several degenerative diseases. Food containing these components is named “functional food”. Citrus fruits contain multiple bioactive agents. The health protective properties of citrus juices have been analyzed in this study regarding some of the most important Citrus species and varieties grown in Sicily. Juices were characterized for their flavonoid profiles and for their antiproliferative activity against additional human cancer cell lines. The important socio economic impact of functional food is discussed.

Key words: Citrus, Flavonoids, Antiproliferative activity, Functional foods,

Socio economic impact of functional food

The concepts of food in the developed world are changing from past emphasis on survival, hunger satisfaction, and absence of the classical nutrient deficiency diseases to an increasingly emphasis on the promising use of foods to promote better health and well-being, thus helping to reduce the risk of chronic illness and conditions such as cardiovascular diseases, some cancers and obesity.

Confusion exists about how to describe this newly evolving area of food and food technology because numerous interchangeable or related terms have been suggested or published in the United States, Europe, and Japan (Table 1). These

include terms such as pharmafoods, functional foods, phytochemicals, chemopreventive agents, and therapeutic foods.

Table 1: *Definitions of health foods and related terminology.*

Term	Definition/characteristic elements
Functional food	Food enriched with ingredients that can improve benefits to health.
Nutraceutical	Is a term that was coined by the American market and it is referred to all consumable food, beverage and supplement products purchased predominantly for health reasons including the prevention and cure of disease.
Phytochemical	Plant components that have health-promoting properties; originally this term was limited to substances found in edible fruits and vegetables that appeared to be protective against cancer. Now the term is much broader and applies to any plant component that has health-enhancing benefits.

Despite these uncertainties regarding the definition of foods with health claims the International Food Information Council (IFIC) named as functional food: “foods that provide health benefits beyond basic nutrition” [International Food Information Council, 2004].

The definition of functional foods excludes supplements such as syrups, pills and capsules. Consumers, however, tend not to differentiate between functional foods and other products with health claims (nutraceuticals) such as supplements, herbal products including herbal medicines.

Nutraceuticals are dealing with a category containing an extremely wide range of products (Fig.1) lacking precise boundaries and which includes natural and organic foods, supplements, functional foods, some lesser evil foods (foods with unhealthy ingredients taken out such us fat, sugar, caffeine, salt, ecc) and

some market standard foods (foods consumed predominantly for health reasons like orange juice, yogurt, fruit and herbal tea and former functional and lesser evil foods that have become the market standard like enriched flour, iodized salt, and low fat milk [Castellini A. et al (2002)].

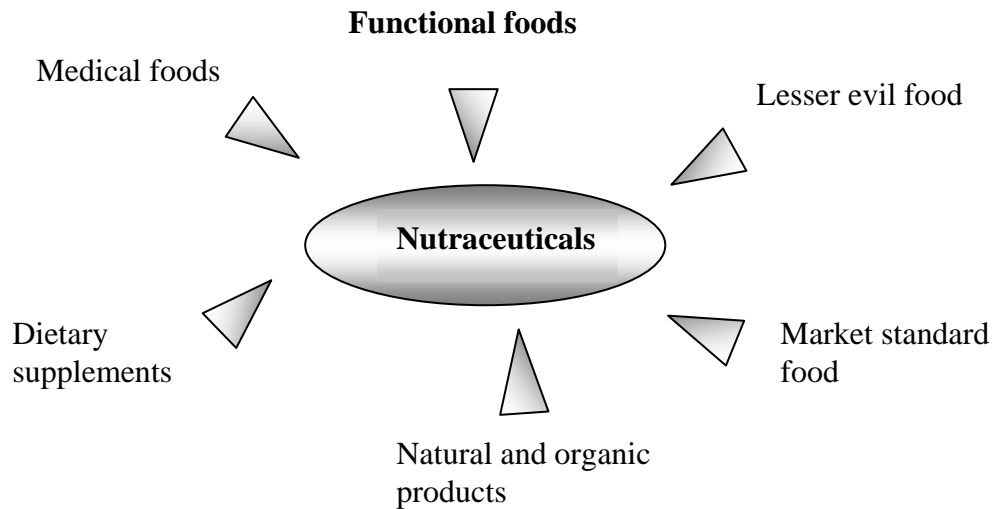


Fig. 1: Nutraceutical categories

In Japan which was the first national authority to establish a specific regulatory framework for functional foods, classed as 'Foods for Specific Health Use' (FOSHU), defined as “Foods which are, based on the knowledge concerning the relationship between foods or food components and health, expected to have certain health benefits, and have been licensed to bear a label claiming that a person using them for specified health use may expect to obtain the health use through the consumption”. FOSHU introduced in Japan in 1991 must be approved by the Minister of Health and Welfare after the submission of comprehensive science-based evidence to support the claim for the foods when they are consumed as part of an ordinary diet.

In February 2000, the total number of approvals under the FOSHU label reached 174 with an estimated market value of around 2 billion US\$. In total more than 1700 Functional Food products have been launched in Japan between 1988 and 1998 with an estimated turnover of around 14 billion US\$ in 1999. [Menrad K., 2003]

In the United States of America, “reduction of disease risk” claims have been allowed since 1993 on certain foods. These contain components for which the Food and Drug Administration (FDA) has accepted there is objective evidence for a correlation between nutrients or foods in the diet and certain diseases on the basis of

`the totality of publicly available scientific evidence, and where there is substantial agreement amongst qualified experts that the claims were supported by the evidence'. [(Ashwell M.(2001)]. Although manufacturers may use health claims to market their products, the FDA's stated intention is that the purpose of health claims is to benefit consumers by providing information on healthful eating patterns that may help reduce the risk of disease such as heart disease and cancer.

Functional foods are products that are removed from the term “food” as understood in more traditional sense and in many aspects are closer to the field of pharmaceuticals. According to the European law they are still only food products and cannot for any reason claim therapeutic or curative claims (this is a property belonging only to medicines). The food product must not be promoted in any way as a treatment for the prevention of pathologies or for the restoration or the modification of a physiological function of the human body. This is a principle that the EU has also hammered in the latest document regarding the subject [European Commission (2001)].

The European Union (EU) has no legal functional food definition, although it developed a working definition where food is called “functional” if it is satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk of disease. Functional foods must remain foods, and they must demonstrate their effects in amounts that can normally be expected to be consumed in the diet. They are not pills or capsules, but part of a normal food pattern. European labelling legislation prohibits attributing to any foodstuff the property of preventing, treating or curing a human disease or referring to such properties. At the same time, there is broad consensus that health claims must be properly substantiated to protect the consumer, to promote fair trade and to encourage academic research and innovation in the food industry.

From a market perspective, functional foods are difficult to quantify because different definitions are used and variability of the different market figures and the forecasts are considerable.

Anyway even if functional foods are still in their nascent state, covering only a few percentage of the total food supply, they are growing rapidly and the global market (inc. food supplements) is estimated by Datamonitor to 73 Billion €(2000), growing by 16% per year. It has also been estimated that the market in US in 1998

was 25 Billion € Europe more than 15 Billion € and Japan 13.5 Billion € (Table 2) [Holm F. (2003)]. Nutrition Business Journal (NBJ Functional Food Report, 2002), has estimated the functional food market in US to 18.5 Billion US \$, the supplement market to 17.6 Billion US \$, and the total nutrition market (inc. the organic foods market) to 55 Billion US \$ in 2002 out of the total food market, estimated to more than 500 Billion US \$ [Holm F., 2003].

Tab 2: Estimate of world functional foods and nutraceutical market (Datamonitor, 1999)

Million US \$	1994	1998	Annual growth rate, %
US			
Functional foods	6.773	12.700	17,5
Food supplements	6.640	9.300	8,1
Total	13.413	22.000	12,8
France, Germany, UK			
Functional foods	6.352	8.511	6,8
Food supplements	3.377	5.155	10,5
Total	9.729	13.666	8,1
Japan			
Functional foods	5.853	8.180	7,9
Food supplements	2.374	3.710	11,2
Total	8.227	11.890	8,9

Leatherhead Food Research Association (LFRA) reports lower market figures due to a more restrictive definition of functional foods. They estimate the global market of functional drinks, to 16 Billion € in 2000 and forecast a 27 Billion € market in 2005, a 70% increase in that period [Krause, C, (2001)].

A food product can be made functional by using any of these 5 approaches:

1. Eliminating a component known to cause or identified as causing a deleterious effect when consumed (eg, an allergenic protein).
2. Increasing the concentration of a component naturally present in food to a point at which it will induce predicted effects [eg, fortification with a micronutrient to reach a daily intake higher than the recommended daily intake but compatible with the dietary guidelines for reducing risk of disease], or increasing the concentration of a non nutritive component to a level known to produce a beneficial effect.

3. Adding a component that is not normally present in most foods and is not necessarily a macronutrient or a micronutrient but for which beneficial effects have been shown (eg, non vitamin antioxidant or prebiotic fructans).

4. Replacing a component, usually a macronutrient (eg, fats), whose intake is usually excessive and thus a cause of deleterious effects, by a component for which beneficial effects have been shown.

5. Increasing bioavailability or stability of a component known to produce a functional effect or to reduce the disease-risk potential of the food.

However, the demonstration of such beneficial effects must be based on science, however to guarantee the credibility of any assertion of benefit because the consumer acceptance is a first prerequisite to market functional foods. This is also true for the attainments of modern biotechnology that may also have a major impact on possibilities to optimise functional foods.

Flavonoids as functional ingredient

The nutritional interest in the significance of free radicals as a risk factor for the incidence of degenerative diseases is only acknowledged for one or two decades. One of the possibilities of 'fighting' free radicals is through the intake of antioxidants. Many bioactive compounds having antioxidant properties have been discovered. Among them, flavonoids, a large class of low molecular weight polyphenolic compounds ubiquitous in plant, deserve the greatest consideration.

Flavonoids have aroused great interest in the last 10-15 years because of their potential beneficial effects on human health. They have been reported to have antiviral, anti-allergic, antiplatelet, anti-inflammatory, antitumor and antioxidant activities so they are considered as "functional ingredients" for their potential role in promoting health and preventing (chronic) diseases.

The health protective properties of these phytochemical components have been demonstrated in a great number of population studies relating flavonoid and isoflavonoid intake to prevention of several degenerative chronic pathologies, including cancer and cardiovascular diseases; these studies have reported an inverse relationship between the assumption of food groups rich in polyphenolic compounds and the incidence of mortality for alimentary cancer and coronary heart disease [Negri et al., (1991); Austoker, (1994)]. The protective role of flavonoids is due to

their antioxidant properties that may protect tissues against oxygen free radicals, mutagenesis and lipid peroxidation. Oxidants may contribute to carcinogenesis both by causing mutations and by stimulating cell division. Many carcinogens, in fact, can generate free radicals that damage cells, setting them to malignant changes. DNA contains reactive groups that are highly susceptible to free radicals attack and oxidative DNA damage can lead to deleterious mutations. Flavonoids act as antioxidants by scavenging free radicals, an activity related to their phenol rings containing hydroxyl groups that reduce free radicals by a donating hydrogen process and cause their removal. The potential of flavonoids to act as antioxidants is dependent on their structural arrangements and, more precisely, on the level of hydroxylation (number of OH groups) and the location of the hydroxyl groups in the primary activity sites.

The structural chemistry determines a hierarchy of flavonoids and isoflavonoid in antioxidant activity, revealed applying a specific redox indicator (TEAC); analysis based on this indicator have shown that flavonoids are more effective antioxidants *in vitro* than vitamins C and E, on a molar basis [Rice-Evans et al., (1997)].

The role of flavonoids has been identified in several biological processes including antiproliferation activity, inhibition of cell cycle, prevention of oxidation, induction of detoxification enzymes, apoptosis, regulation of host immune functions and other mechanisms. The capability to interfere with numerous cellular processes, including growth and differentiation, and to protect against genomic instability suggest that flavonoids may have use as dietary agents in the prevention of cancer and many other degenerative diseases. However, information regarding their presence and bioavailability in foods are still scarce and the comprehension of mechanisms of action of most flavonoids and isoflavonoids with respect to cancer prevention is not clear yet.

The distribution of flavonoids in plants is extremely wide. So far, over 5000 different naturally occurring flavonoids have been described and each plant species is characterized by a particular pattern of compounds. Depending on their structural arrangements, flavonoids are categorized into several subclasses, the most common being: flavonols, flavones, flavanols and isoflavones. These flavonoids often occur as glycosides, glycosylation rendering the molecule less reactive towards free radicals and more water-soluble, so permitting storage in the vacuole [Rice-Evans et al., (1997)]. The sugar most usually involved in the glycoside formation is glucose.

Citrus as source of flavonoids

In spite with their wide distribution, only limited data on their contents in plant foods are available. The genus *Citrus* is considered the major food source of flavanone glycosides, the most abundant components being narirutin, naringin and hesperidin. Their presence and distribution is, however, highly variable, depending on genetic and environmental factors that greatly affect typology and concentration of these bioactive components into different species and varieties.

We have characterized the flavonoid profiles of the most important *Citrus* species and varieties grown in Sicily: Sweet orange (*Citrus sinensis* L. Osbeck) cvs. Washington Navel and Sanguinello comune, Mandarin (*C. reticulata* Blanco), cv Avana; Clementine (*Citrus clementina* Hort. ex Tan.) cv Nules; Grapefruit (*C. paradisi* Macf.) cv Ruby Red and Bitter orange (*Citrus aurantium* sp. *myrtifolia*) cv Chinotto. Fruits were harvested from plants grown in the same location, at the C.N.R. *Citrus* Experimental Field Station of Lascari, Palermo.

The identification and quantification of flavonoids was carried out on fruits harvested at a commercial ripening stage, hand-squeezed and filtered through a stainless steel sieve (1.25 mm) before injection. Juices were immediately processed. High-Performance Liquid Chromatography (HPLC) was used for separation and measurement of *Citrus* flavonoids. Analyses were performed using a HP Agilent 1100 Series, equipped with a 20 µl loop injector (column used: Sepsil C18; UV detector adjusted at a wavelength of 280 nm; the flavanone compounds were identified in the samples by comparing the retention time with that of standards and quantified by comparing the integrated peak areas with that of an internal standard).

The major flavonoids revealed in the *Citrus* juices are indicated in the following table.

Table 3: Quantitative values for juices flavonoids (mg/100 ml of juice); quercetin was used as internal standard.

	eriocitr.	neoeriocitri	narirut.	naring.	hesperidi	neohesperidi	didim.
1	2.400	-	0.214	0.648	5.055	-	-
2	-	-	2.467	-	5.430	-	-
3	7.690	-	7.360	0.560	16.077	-	0.144
4	5.937	-	13.908	-	30.798	-	2.922
5	-	13.634	-	18.593	-	14.270	4.714
6	-	-	54.20	128.00	7.13	7.42	6.72

1 - Avana mandarin; 2 - Nules clementine; 3 – Sweet orange Washington Navel; 4 – Sweet orange Sanguinello comune; 5 – Chinotto Bitter orange; 6 - Ruby Red Grapefruit.

Knowledge of the Citrus flavonoid content is paramount to understanding their role in human health. Several *in vitro* and *in vivo* experiments, in fact, have shown that flavonoids could inhibit initiation, promotion and hyperproliferation of cancer cells [Hollman et al., (1996); Manthey and Guthrie, (2002)]. The role of flavonoids, however, in human cancer prevention is in the process of being defined, their mechanisms of action being not clear yet, varied, complementary and/or overlapping [Birt et al., (2001)]. Considering, in fact, the great variety of bioactive components present in *Citrus* fruits, it appears extremely unlikely that only one category of substances, even if very abundant in juices, is responsible for the associations seen between *Citrus* foods and cancer prevention. Most probably, the health protective properties of *Citrus* juices derive from the interaction between multiple dietary chemopreventive agents, flavonoids and isoflavonoids included.

To further demonstrate the potential health-promoting properties of *Citrus* juices, we carried out a screening of the juices extracted from the fruits of the species previously characterized for their flavonoid content, by measuring their *in vitro* antiproliferative properties against three common human cancer cell lines and precisely: K562 (human chronic myelogenous leukemia), HL-60 (human leukemia) and MCF-7 (human breast adenocarcinoma). The cell lines were grown at 37 °C in a humidified atmosphere containing 5% CO₂, in RPMI-1640 medium or MEM (Sigma) in the case of MCF-7, supplemented with 10% fetal bovine serum and antibiotics.

The *in vitro* antiproliferative activity of the fruit juices was tested in the following way: K562 cells were suspended at a density of 1×10^5 cells/ml, HL-60 cell line was suspended at 2×10^5 cells/ml in growth medium, transferred to 24-well plate (1 ml per well), cultured with or without (in the case of control wells) a screening volume of 100 μ l of fruit juices and incubated at 37° C for 48 h. Control wells were added with 100 μ l of a citric acid solution 1% w/v whose pH was comparable to pH of fruit juices.

Numbers of viable cells were determined by counting in a hemacytometer after dye exclusion with trypan blue.

The antiproliferative activity against MCF-7 was determined by MTT (methyltetrazolium) assay. The experiment was carried out as follows: cells were suspended at a density of 2×10^5 cells per ml in MEM, supplemented with 10% fetal calf serum and antibiotics, transferred (100 μ l per well) to 96-well plate and incubated at 37 °C for 4 days until the formation of cellular monostrate. After this time, the original medium was replaced with RPMI without red phenol and a juice fruit screening volume of 10 μ l was added to each well and incubated for three days.

The antiproliferative effects of the fruit juices were estimated in terms of percent growth inhibition comparing cell viability of treated and untreated cells (but added with 10 μ l of a citric acid solution 1%) by their reduction of the tetrazolium substrate (MTT) to formazan.

Many of the *Citrus* juices exhibited antiproliferative activity toward the cancer cell lines K562, HL60 and MCF-7. The cellular growth inhibition percentages are reported in the following table.

Table 4: Growth inhibition percentage recorded at screening concentration of 10% v/v.

Juice	K562	HL-60	MCF-7
Wash. Navel	82.6	73	Ns
Nules	39.7	Nt	Ns
Bitter orange	100	100	84.3
Avana	43.4	Ns	Ns
Sanguinello	100	100	90.5
Ruby Red	100	100	89.2

Values are the mean of at least three independent determinations; coefficient of variation was less than 15%; ns = not significant i.e. below 10% inhibition; nt = not tested.

The genotypes exhibiting the strongest antiproliferative activities *in vitro* at 10% juice v/v concentration were Sanguinello comune sweet orange, Chinotto bitter orange and Ruby Red grapefruit. Their levels of growth inhibition occurred at very similar values with regard to all the different tumoral cell lines. A weaker activity was observed for the juice of Washington navel sweet orange, which was inactive toward the MCF-7 tumoral line. The lowest *in vitro* inhibitory activity were observed for the Avana mandarin and for the Nules clementine, both being inactive as antiproliferative agent toward the MCF-7 cellular line. The human breast adenocarcinoma MCF-7 cell line was the least susceptible to the cytotoxic effects of the *Citrus* juices.

The three genotypes exhibiting the most significant antiproliferative activities at 10% juice v/v concentration - Sanguinello comune sweet orange, Chinotto Bitter orange and Ruby Red grapefruit - were tested at lower concentrations, 5% and 3% v/v juice concentration. Data are reported in table 5. As expected, the cellular growth inhibition percentages were reduced. The Ruby Red grapefruit and Sanguinello comune sweet orange exhibited, at 5 v/v juice concentration, high levels of cell growth inhibition toward the K562 and HL60 tumoral lines; their antiproliferative activity occurred at similar values. The Chinotto bitter orange showed low levels of activity toward the three tumoral cell lines. Only the Ruby Red grapefruit exhibited high levels of antiproliferative activity toward the MCF-7 cancer cell lines.

Relatively low levels of growth inhibition were exhibited when the three *Citrus* genotypes were applied at 3% v/v fresh juice concentration. Only the Ruby Red grapefruit exhibited significant activity at this concentration toward the MCF-7 cell line, even if at a low level.

Table 5: Growth inhibition percentages of samples recorded at 5 %, and 3 % v/v concentrations.

Fruit		K562	HL-60	MCF-7
Bitter orange	5%	33.7	31.7	Ns
	3%	Ns	Ns	ns
Sanguinello	5%	95.1	80.1	23.6
	3%	36.1	29.4	Ns
Ruby Red	5%	96.7	95.1	88.5
	3%	38.4	33.6	19.4

Values are the mean of at least three independent determinations; coefficient of variation was less than 15%; ns = not significant i.e. below 10% inhibition

Among the *Citrus* genotypes tested for their *in vitro* antiproliferative activity, the most effective juices were those from Sanguinello sweet orange and Ruby Red grapefruit. Both these varieties belong to *Citrus* species characterized by a wide genetic variability (group of “blood oranges” for the Sanguinello comune and group of “pulp pigmented” grapefruit for the Ruby Red), therefore their flavonoid profiles, as well as their antiproliferative activity, might vary consistently. In order to investigate on the relationship between flavonoids and positive health effects, we have characterized the flavonoid profiles of different varieties belonging to the two species *Citrus sinensis* and *Citrus paradisi*, taking into account only the ‘pigmented’ varieties for the sweet oranges and also the ‘not pigmented’ varieties for grapefruit (table 6).

Table 6: Flavonoid contents (mg/100 ml) in different varieties of *Citrus sinensis* and *Citrus paradisi*.

	ERIOC.	NEOERIOC.	NARIRUTIN	NARINGIN	HESPERIDIN	NEOHESPERID.	DIDIMIN	QUERCETRIN
1			27.49		94.86		12.16	
2			12.88		69.95		4.93	
3			2.80		11.43		1.16	
4			20.17		60.88		5.30	
5			7.56		34.73		3.62	
6	7.22	4.21	11.15	24.50	2.13	12.86	1.56	2.27
7			14.16	28.76	2.03	2.47	2.39	4.32
8			23.23	59.58	3.00	4.35		4.96
9			54.20	128.00	7.13	7.42	6.72	14.80
10			22.11	68.20	3.10	5.63		5.86
11			12.25	35.48	1.79	2.63		4.31

Blood oranges (*Citrus sinensis* L. Osb.) cvs :

1 - Sanguinello; 2 - Moro; 3 – Tarocco Scirè; 4 – Tarocco Meli; 5 – Tarocco Gallo;

Grapefruit (*Citrus paradisi* Macf.) cvs :

6- Imperial (n.p); 7- Foster (n.p.); 8-Duncan (n.p.); 9- Ruby Red (p.); 10- Red Blush (p.); 11- Tompson (n.p.).

n.p. = not pigmented pulp; p. = pigmented pulp

High variability in flavonoid typology and concentration has been observed among the genotypes belonging to the two *Citrus* species. Of great interest is the high amount of hesperidin in Sanguinello sweet orange and naringin in Ruby Red grapefruit, both constituting more than 70% of total flavonoids of the relative juice. In grapefruit, naringin is accompanied by narirutin (naringenin 7- rutinoside). The activity of hesperidin, naringin and naringenin has been pharmacologically evaluated in terms of chemoprevention of carcinogenesis, inhibition of human cancer cell proliferation and delay of tumorigenesis [Tanaka, (1997); So et al., (1996)].

Accordingly, in a preliminary experiment we have assayed the *in vitro* antiproliferative activity of these compounds on NCI-H460 (human lung cancer) cell lines; naringenin exhibited significant inhibitory effects (92.5% inhibition at 100ul/ml concentration), while naringin did not cause a significant inhibitory effect, at the same concentration. The *in vitro* antiproliferative activity of naringenin, could suggest its potential as useful anticancer agent, on condition that the same effect is confirmed following *in vivo* testing. Further experiments are, in fact, needed to clarify if *Citrus* juice can act as an anticancer *in vivo*, however, it could be speculated that the supplementation of the natural antitumoral compounds contained in the *Citrus* juice through a balanced diet might represent an important step in a general cancer prevention strategy.

The health-protective properties of juices could be further increased if citrus fruits showing new and more effective combinations of antitumoral components are identified. Attempts in this direction are in course using a biotechnological approach in our laboratory and the goal is to produce and select new citrus genotypes showing useful traits for disease prevention and health protection. Following somatic hybridization by protoplast fusion [Grosser and Gmitter, (1990)], several somatic hybrids have been obtained; these genotypes combine the genomes of citrus species and varieties showing effectiveness against cancer cell proliferation (sour orange and several clones of sweet oranges, grapefruits and also lemon). Hybrids have not fruited in a consistent manner yet, but preliminary analysis on few fruit samples show new combinations of bioactive compounds, in a special way flavonoids and isoflavonoids, as compared to the parental plants. Test on the antiproliferative activity of these juices are underway at the moment.

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