

Biochemical Characteristics, Antimicrobial and Mutagenic Activity in Organically and Conventionally Produced *Malus domestica*, Annurca

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Abstract: Total polyphenols content and a partial phenolics composition from pulp and peel of organically and conventionally produced *Malus domestica*, Annurca were investigated. Furthermore, the antimicrobial effect of these compounds against useful and pathogen microorganism was examined. Finally, their mutagenic activities were evaluated. Organically grown apples showed a greater content of polyphenols, both into the pulp and the peel. The distribution of polyphenols was different between the two types of fruit, the organic fruit showing a more concentration of them, mainly into the peel extracts. The antimicrobial activity was also different: the conventionally produced samples showed a good inhibitory effect against *Bacillus cereus*, the organic peel revealed in addition a good inhibitory effect against *Escherichia coli* 0157:07. The results demonstrated a probable relationship between the phenolic composition and distribution in the extracts and the antimicrobial effect. The Ames test did not reveal the presence of an evident mutagenic activity in the conventional extracts. The antimutagenic activity was more evident in the organic extracts.

Keywords: Ames test, Annurca, Antimicrobial, phenolics, HPLC, *Malus domestica*.

INTRODUCTION

Apples are worldwide diffused fruit with many health beneficial effects, mostly due to the presence of phenolic compounds. Polyphenols are common secondary metabolites of plants, with a well known putative role in protection against the infection by plant pathogens [1]. Apples also ranked the second for total content of phenolic compounds, including quercetin, catechin, phloridzin and chlorogenic acid, all of which are strong antioxidants, and thus capable of counterbalancing free radical activities that may cause cell injuries [2-3].

The phytochemical composition varies greatly among different varieties of apples. The importance of plants as natural sources of phenolics is supported by epidemiological studies, which generally link the consumption of polyphenols with a reduced risk of some cancers, cardiovascular disease, asthma, and diabetes. Furthermore, these metabolites show other interesting biological properties, such as anti-inflammatory, antioxidant, antiviral and antimicrobial activities [4-5]. Interest in the antimicrobial properties of phenolics is strengthened by suggestions that they affect the behaviour of pathogenic bacteria in foods derived from plants; indeed, their use as natural additives in food industry is increased since last years [6].

Annurca apple (*Malus domestica*, Annurca) is one of the most important and ancient cultivars in Campania region, Southern Italy, and it is the unique to have received from the European Council the "Protected Geographical Indication", related to the preservation of local and characteristics agriculture commodities (CEE rule nr 2081, 1992). Typical elements which differentiate the Annurca from the other varieties are the traditional method of growing and manufacturing

as well as its morphological aspect of its pomace. Fruits are treated in a particular manner to obtain their typical redness. After harvest, they are placed for about 30 days on a layer of straw on the soil and daily sprayed with water. When the surfaces of fruits become red, apples are manually turned so to be homogeneously reddened. Typical Annurca is an apple of middle size, with a weight of about 100 g; its peel has a dark red colour with yellow areas and, on the top, a characteristic and no more wide rusty area. Pulp is white, crunchy, sweet and lightly acidic, with a pleasant aroma and taste. Many studies were performed on different aspects of its biology and biochemistry, concerning texture composition, aroma and the identification of several polyphenols [7-9].

The aim of our work was to highlight some biochemical, microbial and genotoxic characteristics of extracts from organically and conventionally produced Annurca apple. In essence, we examined the polyphenol content and performed a partial composition in polyphenols present in pulp and peel as well the antimicrobial activity of these extracts against unwanted, pathogen or lactic acid bacteria. Finally, we analysed their *in vitro* mutagenic properties.

MATERIALS AND METHODS

Standards and Reagents

All standards were obtained from Sigma Chemicals (St Louis, MO). Acetonitrile and trifluoroacetic acid were obtained from Carlo Erba Reagenti (Milan, Italy). Acetone, methanol, ethanol and ethyl acetate were purchased from Sigma Chemicals. All reagents were of analytical grade.

Fruit Material

The experiments were performed on commercial samples of organically and conventionally produced samples of *Malus domestica*, Annurca. Immediately on arrival in laboratory, fruits were washed; pulp and peel were accurately separated and stored at -26°C until the extraction of polyphenols.

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Preparation of Extracts

The extracts were prepared following partially the method of Fratianni, Tucci, De Palma, Pepe & Nazzaro [10]. Briefly, frozen samples were incubated for 1 h at 4°C in 5 volumes of acetone: ethanol: methanol (70:15:15), avoiding direct contact with light, concentrated by drying and re-suspended in methyl alcohol. The concentrates were filter-sterilized (0.22 µm membrane) and kept at -26°C.

Total Phenolic Assay

Total polyphenol content was measured using the Folin Ciocalteu colorimetric method [11]. To 800 µL of deionised water, 50 µL of Folin Ciocalteu's phenol reagent and a volume of sample ranging from 10 to 50 µL were added and accurately mixed. After 1 min, 100 µL of 20% sodium carbonate solution was added and mixed. Deionised water was then added up to a volume of 1 mL. The solution was carefully mixed and total phenols were spectrophotometrically estimated at 765 nm (DU-Beckman, USA) after 2 h incubation. Quantification was based on the standard curve generated with quercetin. All determinations were carried out in triplicates.

Chromatographic Analysis

All standards used in the experiments were accurately weighed, dissolved in methanol and treated with ultrasonic waves for 10 min. The calibration curves were generated with concentrations ranging from 0.01 to 0.5 mM of chlorogenic acid, coumaric acid, ferulic acid, caffeic acid, catechin, epicatechin, phloridzin. The chromatographic analysis of the apple extracts was performed [10] by RP-HPLC, using a Gold System apparatus equipped with an UV detector (Beckman, CA, USA). A Khromasyl KR 100-5 C-18 column (25 cm x 4.6 mm) at room temperature was used for this analysis. The mobile phase included HPLC pure water (containing 0.1% trifluoroacetic acid, solvent A) and 95% acetonitrile (containing 0.1% trifluoroacetic acid, solvent B) in the following gradient system: initial 10% B, linear gradient to 50% B in 30 min, linear gradient to 100% B in 5 min, hold at 100% B for 2 min, decreasing gradient to 10% B in 2 min and coming back to the initial steps 10% B for 10 min. The total pre, running and post running time was 54 min. The flow rate was 1 ml/min, the injection volume was 20 µl, and the detection wavelength was set at 280 nm.

Microorganisms and Culture Media

Lactobacillus casei DSM 9595, *Lactobacillus fermentum* DSM 20052, *Lactobacillus acidophilus* DSM 20079, *Lactobacillus bulgaricus* DSM 20081, *Lactobacillus rhamnosus* DSM 20711, *Escherichia coli* DSM 8579, *Escherichia coli* EC 101, *Bacillus cereus* DSM 4313, *Bacillus cereus* DSM 4384, and *Bacillus cereus* GN 105 were grown for 18 h into specific growth media: Lactic acid bacteria in MRS broth (OXOID), *Escherichia coli* and *Bacillus cereus* in Nutrient Broth (OXOID).

Antimicrobial Activity

Antimicrobial activity was performed by the inhibition halo test on agar plates. The microbial suspensions (1×10^8 CFU/ml) were uniformly spread onto the specific solid media plates. Sterile Whatman n°1 filter paper discs (5mm in diameter) were individually placed on the inoculated plates

and impregnated with polyphenolic extracts (38 µmol). After 30 min standing in sterile conditions at room temperature, plates were incubated for 24-48 h at 37°C, depending on the strain. The diameter of the clear zone shown on plates was accurately measured and expressed in millimetres as its antimicrobial activity. Sterile deionised water was used as negative control; the standard antibacterial agent, chloramphenicol (8 µg), was used as a positive control [12]. All antimicrobial experiments were performed in triplicate using independently-prepared plates.

Mutagenicity Test

For mutagenicity assay on extracts from Annurca apple, the Ames assay was performed [13] by using the *Salmonella typhimurium* histidine-requiring strains TA98 and TA100, purchased from Molecular Toxicology Inc. (Moltox™, Annapolis, MD, USA). Strains were aerobically grown at 37°C in Nutrient Broth n° 2 (OXOID) supplemented with ampicillin. The test was carried out by addition of 0.1 ml of the overnight bacterial culture, polyphenol extracts (32.2 mmol), and 0.5 ml phosphate buffer (0.1 M, pH 7.4) to 1.8 ml of molten agar; the mixture was immediately plated onto minimal medium agar plates (previously added with 0.2 ml of sterile 0.5 mM Bio-His) and incubated at 37°C for 48 h in the dark. Positive and negative (spontaneous revertants) controls in the presence and absence of the diagnostic mutagen were included. As positive controls, the direct acting mutagens nitrofluorene was used for strain TA 98 and sodium azide for strain TA 100; the indirect acting mutagen was 2-aminoanthracene. Metabolic activation was achieved [13,14], by a polychlorinated biphenyl mixture Aroclor 1254-induced S9 homogenate (0.7 nmol cytochrome P450/mg protein) prepared from male Sprague-Dawley rats and incorporated in the S9 mixture (Moltox™, Annapolis, MD, USA). All tests were conducted in triplicate and the results averaged.

Antimutagenicity Testing

Antimutagenicity testing procedures were essentially as described by Ames and coworkers [13]. Briefly, the mutagen standard was added to the mixture of pulp or peel apple extract (32.2 mmol), bacterial culture, with S9 mix (indirect test) or phosphate buffer (direct test). After incubation for 30 min at 37°C, the mixture was added to 2.0 ml of molten agar and plated as above. Positive and negative controls were also included in each assay. His+ revertants were counted after 48 h of incubation at 37°C. The antimutagenic activity was expressed as percentage of inhibition: $[1 - (T - S/M - S)] \cdot 100$, where T is the number of revertants/plate in the presence of mutagen and the test sample, M is the number of revertants/plate in the positive control and S is the number of spontaneous revertants/plate [15].

RESULTS AND DISCUSSION

Total Phenol Content

The polyphenol content of pulp and peel of organic and conventional Annurca samples is presented in Table 1. Pulp and peel showed a different phenolic content: the value evidenced in the peel was found to be 14 times superior than in corresponding pulp. Because the apple peels contain more antioxidant compounds, they may have higher antioxidant activity and higher bioactivity than the apple pulp. Research

demonstrated that apples without the peels had less antioxidant activity than apples with the peels. Apples with the peels were also better able to inhibit cancer cell proliferation when compared to apples without the peels [5]. Other studies have shown that apple peels contain anywhere from two to six times (depending on the variety) more phenolic compounds than in the flesh. The antioxidant activity of these peels is also much greater, ranging from two to six times greater in the peels when compared to the flesh, depending on the variety of the apple [16]. By focusing on the phenols in two types of Annurca apple, we found that the organically grown variety contained many more phenols in contrast to those grown in a conventional way. A different content in polyphenols was also reported by Veberic *et al.* [17] and Hecke *et al.* [18], which studied several organic and integrated varieties of apple. Generally, the content of polyphenols may be expected in vegetable organisms also as reaction against biotic or abiotic stress. The application of pesticides and fertilisers has been previously reported to affect the biosynthesis of phenolics in plants, substituting their action, so that conventionally grown plants generally show a phenol content lower than the same but organically grown varieties [19,20].

Table 1. Total Polyphenolic Content in Pulp and Peel Extracts of Organic and Conventional Annurca Apple

	Pulp	Peel
Organic apple	66.88 (3.67)	941.17 (26.77)
Conventional apple	12.29 (3.98)	267.09 (19.53)

Data are mean values of 3 three determinations and are expressed as $\mu\text{mol/g}$ of fresh product. SD values are shown in parenthesis. For details: see materials and methods.

HPLC Analysis

Usually, the compounds most commonly found in apple peels consist of the catechin, epicatechin, procyanidins, chlorogenic acid, phloretin glucoside (phloridzin). In the apple flesh, there is some catechin, procyanidin, epicatechin, and phloridzin but these compounds are found in much lower concentrations than in the peels [21]. Above all, we analysed the polyphenols ferulic, chlorogenic *p*-coumaric and caffeic acid; the content of flavan-3-ols catechin and epicatechin as well as phloridzin was also investigated. The chromatographic analysis of some polyphenols is reported in

Table 2. Generally, organic Annurca showed a greater concentration of the polyphenols considered in our analysis. In the organic peel, the most abundant compounds were found to be catechin and epicatechin, as well as chlorogenic and caffeic acid, all of them generally present at a concentration of about or more than ten times in comparison to the conventional ones. On the contrary, the distribution of *p*-coumaric and ferulic acid was different, being the two polyphenols much more abundant in the conventional peel. The content of phloridzin was similar in the two extracts, being more abundant only in the organic peel extract. The presence of catechin and epicatechin, mainly in the organic apple, is important due to their strong antioxidant activity [22]. Furthermore, the good percentage of *p*-coumaric acid found in the organically and conventionally produced Annurca could be significant since this biomolecule has received a lot of attention in the recent years for its anti-inflammatory *in vivo* properties [23]. Moreover, the high level of chlorogenic acid, observed mainly in organic fruits is of great meaning as *in vivo* studies have demonstrated the antioxidant and anticarcinogenic properties of this biomolecule, in general inefficiently absorbed in small intestine, but capable of providing higher yields of microbial metabolites, active compounds responsible for the biological properties attributed to dietary polyphenols [24-25]. The characterization of conventional and organic apple aimed at the identification of compounds developed by plant tissues as part of their defence mechanism was carried out by Lucarini *et al.* [26], which indicated higher levels of both polyphenols and specific phenolic acids, as *p*-coumaric and caffeic acid, in organically than in conventionally produced apples. On the whole, our results were similar relating to the total polyphenol content and to caffeic acid; they were also in agreement with the high content of chlorogenic acid found in apples [27] but could be shortly divergent with other researches performed on Annurca apple [9], in which the concentrations of some polyphenols, such as chlorogenic acid was different. Perhaps, this different content could be ascribable to a different time of harvesting of Annurca apple or to the different geographical area of growing.

Antimicrobial Test

Since many years, phenolic compounds became the subject of anti-infective research, due to their antibacterial, antifungal and antiviral activity [28-31]. They can have an effect on growth and metabolism of bacteria, activating or inhibit-

Table 2. Chromatographic Results of Polyphenols Analysis in Pulp and Peel Extracts of Conventionally and Organically Grown Annurca Apple

	Conventional Pulp	Conventional Peel	Organic Pulp	Organic Peel
Chlorogenic acid	9	18	11	118,9
Caffeic acid	7,5	9	14,05	131
<i>p</i> -Coumaric acid	1,4	100,05	2	70,9
Epicatechin	16	35,2	19,15	305,2
Ferulic acid	0,89	71,05	1,45	12
Catechin	2,5	4,2	4,1	137,6
Phloridzin	7,19	5,28	5,56	16,88

The data are expressed as $\mu\text{mol/g}$ of fresh product. Values represent the mean of three different analyses of three independent samples. For details: see materials and methods

ing microbial growth according to their constitution and concentration [32]. We have analysed the effect of different concentrations (from 9 to 38 μmol) of apple Annurca extracts on different useful (*Lb. acidophilus*, *Lb. bulgaricus*, *Lb. casei*, *Lb. fermentum*, *Lb. rhamnosus*) or pathogen microorganisms of agro-food or human interest, such as *B. cereus*, an aerobic spore-former commonly found in raw and processed foods, which determines several cases of food-borne illnesses, and the EHEC entero-haemorrhagic *E. coli* DSM 8579, that causes serious cases of food poisoning. None of apple extracts assayed gave positive results against the 6 strains of lactic acid bacteria. The conventional extracts determined the formation of inhibition halos, having a diameter of about 10mm, only against the three strains of *B. cereus* GN105, DSM 4313 and DSM 4384, at 38 μmol ; a more marked action was evident only against *B. cereus* GN105 with the maximum of extracts tested (inhibition halo =18 mm). The extracts from the organically grown apple exhibited a clear antimicrobial activity, both against the strains of *B. cereus* and against the two strains of *E. coli*. In this case, peel was found to possess a discrete activity, the inhibition halos increasing from 6 mm (with 19 μmol of extracts tested on *B. cereus* DSM 4313) to 13mm of diameter (against *E. coli* EC 101 treated with 38 μmol). The effectiveness found against the pathogenic microorganism *E. coli* DSM 8579 serotype O157:H7, an EHEC enterohaemorrhagic *E. coli*, of worldwide interest for the human health, can be considered of promising importance, as this strain produces verocytotoxins, and causes haemorrhagic colitis and haemo-

lytic uremic syndrome. In our experiments, the inhibition halos showed a diameter ranging from 7 mm to 11 mm) (by testing 19 μmol and 38 μmol , respectively). The inhibition halo caused by chloramphenicol was of 28 mm. Previous reports on the antimicrobial activity of phenolic acids were described, relatively to the growth inhibition of specific polyphenols and to the different species of apple: a slight or no activity was found, by using extracts from the whole fruit of a Finnish apple variety, against *S. aureus*, *S. epidermidis*, *B. subtilis*, *M. luteus*, *E. coli* [33]; on the contrary, an effective activity of extracts mainly from Granny Smith apple, and less from Royal gala, was observed against *E. coli*, *P. aeruginosa* and *S. aureus* [34]. Our results reported, for the first time, a good antimicrobial activity exhibited from Annurca extracts. However, the different activity of organic and conventional apples against the pathogenic bacteria tested, let us hypothesise a strong relationship between content and distribution of phenolics and antibacterial effect.

Mutagenicity Assay

It is well known that a large number of compounds that are *in vivo* carcinogenic also produce mutagenicity in the *in vitro* Ames test of mutagenicity, producing a direct or an indirect (promutagenic) activity [13]. Table 3 shows the results obtained from the Ames test by using *S. typhimurium* TA 98 (Table 3A) and *S. typhimurium* TA 100 His- strains (Table 3B) to detect the direct and indirect mutagenic

Table 3. Enumeration of His+ Revertants in Strain TA 98 (3A) and Strain TA 100 (2B) in the Ames Test

Table 3A.

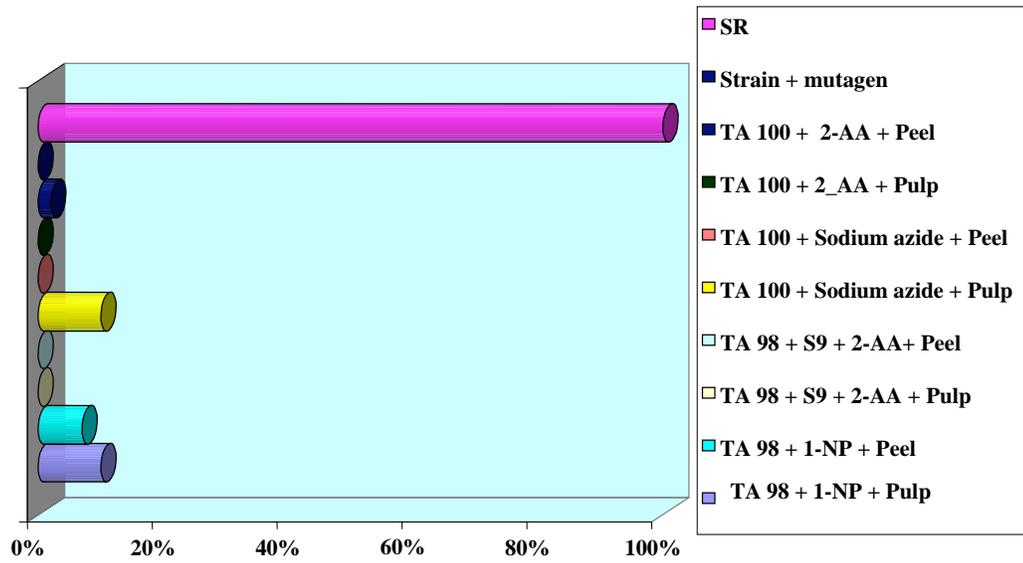
TA 98 His + Spontaneous Revertants (Normal Values: 20-50)	CFU/Plate - S9 Mean (SD)	CFU/Plate + S9 Mean (SD)
TA 98 Negative control (with phosphate buffer)	34 (6.50)	40 (11.01)
TA 98 Positive control (with mutagen standard)	266 (13.01)	200 (18.03)
TA 98 + extract from peel of organic apple (32.2 mM)	24 (3.60)	20 (10.01)
TA 98 + extract from pulp of organic apple (32.2 mM)	25 (4.58)	21 (4.72)
TA 98 + extract from peel of conventional apple (32.2 mM)	30 (7.81)	154 (29.46)
TA 98 + extract from pulp of conventional apple (32.2 mM)	39 (4.58)	152 (14.00)

Table 3B.

TA100 His + Spontaneous Revertants (Normal Values: 80-200)	CFU/Plate -S9 Mean (SD)	CFU/Plate + S9 Mean (SD)
TA 100 Negative control (with phosphate buffer)	135 (19.31)	110 (21.59)
TA 100 Positive control (with mutagen standard)	366 (17.61)	400 (21.93)
TA 100 + extract from peel of organic apple (32.2 mM)	114 (9.86)	90 (10.81)
TA 100 + extract from pulp of organic apple (32.2 mM)	95 (7.93)	105 (24.26)
TA 100 + extract from peel of conventional apple (32.2 mM)	85 (14.73)	185 (15.71)
TA 100 + extract from pulp of conventional apple (32.2 mM)	84 (6.55)	193 (13.52)

The data represent the mean of three independent experiments. Each experiment was performed in triplicate. SD values are shown in parenthesis. CFU: Colony Forming Units. For details: see materials and methods.

A. Antimutagenicity Tests on Conventional Annurca apple



B. Antimutagenicity Test on Organic Annurca Apple

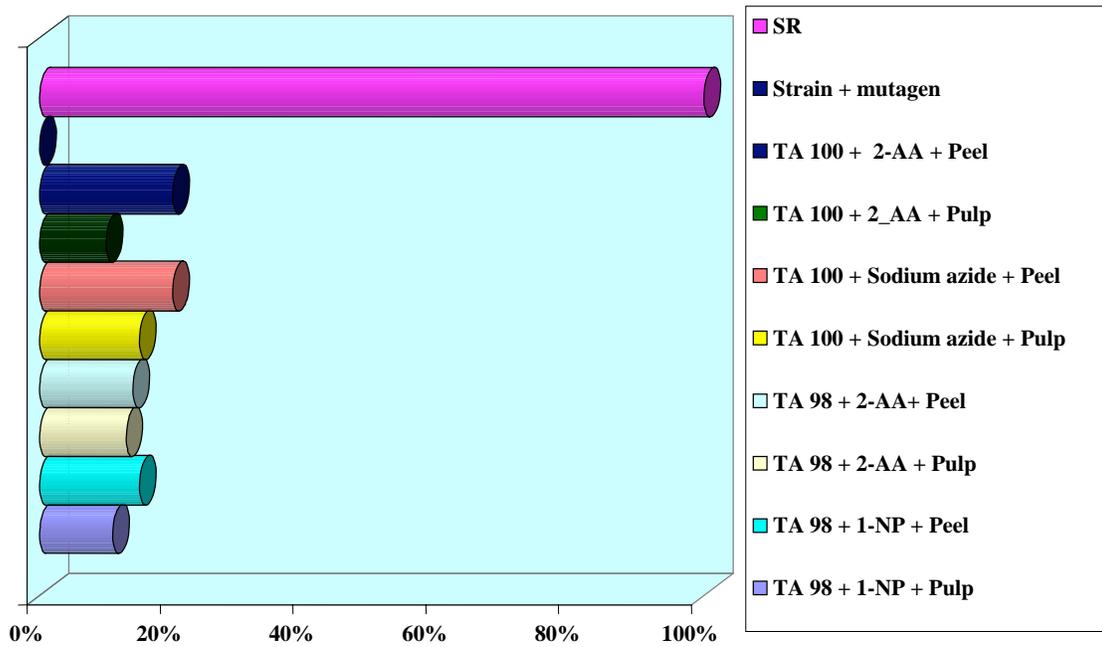


Fig. (1). Antimutagenic activity of extracts from pulp and peel of conventional (A) and organic (B) apple, Annurca, against nitrofluorene (-S9) and 2-aminoanthracene (+S9) induced mutagenesis in *S. typhimurium* TA98 and sodium azide (-S9) and 2-aminoanthracene (+S9) induced mutagenesis in *S. typhimurium* TA100. The results are the mean of three independent experiments and are presented as percentage of inhibition of mutagenesis. Each experiment was performed in triplicate. SR: Spontaneous Revertants. For details: see materials and methods.

activity of pulp and peel extracts when a very high concentration (32.2 mmol) of polyphenols was used. The extracts from conventional apple showed, in the direct test with TA 98, values very similar to those recorded in absence of the mutagen standard, with a number of CFU/plate ranging from 30 to 39. However, in the indirect test, the number of CFU/plate, even if not reaching the values of the positive control, was higher if compared to the negative control (Table 3A); the same trend was observed when the extracts from pulp and peel from conventional apples were put in contact with *S. typhimurium* TA 100, both in the direct and in the indirect test (84-85 versus 135, and 198-185 versus 110 CFU/plate respectively, Table 3B). The organic extracts gave values always lower to the positive control and very similar to the negative control both in the direct test and in the indirect test with TA 98, (Table 3A,B). In some occasions, values of CFU/plate were also lower than the negative control (Table 3A). So, the Annurca apple extracts did not induce evident gene mutations, this behaviour being more marked manner for the organic extracts and less evident for the conventional ones, probably due to a different concentration and distribution of polyphenols between conventional and organic apple and between the different portion of the fruit; this is in agreement with previous works, which demonstrated the no toxicity of some polyphenols, such as catechins and procyanidins in mutagenicity test, and chlorogenic acid, which is the main phenolic acid derivative in apple [35-37].

Antimutagenicity Test

Fig. (1) represents the inhibitory effects exhibited by extracts of conventional (1A) and organic (1B) pulp and peel extracts against nitrofluorene (direct acting) and 2-aminoanthracene (indirect acting) mutagenesis (for *S. typhimurium* TA98) and sodium azide (direct acting) and 2-aminoanthracene (indirect acting) mutagenesis. Organic samples showed always a whole inhibitory effect against the mutagen standard, with percentages of inhibition until 20% for peel. The extracts from conventionally produced apples did not cause such an effect in so evident way, giving rare percentage of inhibition (until 15%) only for pulp. The different action performed by organic and conventional apples Annurca could be ascribable not only to the different practise of growth, but also to the different concentration of some phenolics, such as chlorogenic acid, catechin and epicatechin, with well known antimutagenic activity [36,37], which we found to be more concentrated in the organic apple.

CONCLUDING REMARKS

Taken together, this study demonstrated a difference, in terms of biochemical distribution and concentration of some polyphenols of interest, and a different antimicrobial behaviour of phenolic extracts against some pathogen bacteria, between organic and conventional Annurca apple. Use of peel extracts from organically grown Annurca apples could be considered of great pharmacological interest, due to their antimicrobial activity against some food-borne bacteria such as the enterohemorrhagic *E. coli* 0157:07. The results did not reveal the presence of components in conventional Annurca apples that could be clearly mutagenic in the Ames test, thus indicating that the conventional growing practise did not lead the formation of mutagenic compounds. However, the different behaviour observed from the extracts from conventional

fruit, mainly in the indirect test, a situation that mimics the contact between the possible promutagenic compounds and the human liver, let us hypothesise how the different composition and distribution of the phenolic compounds, could give a relative loss or modification of some constituents which could have initially acted as antimutagenic. Therefore, this hypothesis could be supported by the results observed in the antimutagenicity test. The organically produced fruit excludes the presence of some synthetic substances; it could also guarantee a more balanced product, in terms of polyphenol distribution, in possess also of better nutritional, biological and toxicological characteristics. Further studies are planned to deeply investigate the antimicrobial and antimutagenic activities of Annurca extracts and to attempt to isolate their bioactive components.

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