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Effect of different extracting solvents on antioxidant activity and phenolic compounds of a fruit and vegetable residue flour

Mônica C. P. Santos; Édira C. B. A. Gonçalves*

Department of Food Technology, Nutrition School, Food and Nutrition Master Program, Federal University of Rio de Janeiro State - UNIRIO. Av. Pasteur, 296, Urca, 22290-240, Rio de Janeiro, Brazil. Phone: (+55) 21 2542 7418.

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Abstract

In order to quantify antioxidant capacity in food products, several methods have been proposed over the years. Among them, DPPH radical is widely used to determine the antioxidant capacity of different substrates. However, it is known that different types of extractants, providing different responses, can extract a variety of bioactive compounds. Besides, storage time seems to interfere in the stability of these substances. Integral use of fruits and vegetables has been proposed along the years as a means of reducing environmental pollution and give a better destination to by-products from food industries. Thus, this study aimed to evaluate the antioxidant potential of a fruit and vegetables residue flour (FVR) with sequential and non-sequential extraction, in order to evaluate its antioxidant activity and phenolic compounds. And these compounds stability during storage of 180 days. It was observed that in non-sequential extraction, water was able to reduce by 74% the radical; however, at sequential extraction process, using six different extractors, each one was able to reduce at least 40% of DPPH. The total soluble phenolic contents in sequential extraction were 22.49 ± 1.59 mg GAE/g FVR on the first day and 5.35 ± 0.32 mg GAE/g FVR after 180 days.

Keywords: antioxidant activity; phenolic compounds; residue flour; DPPH assay.

Chemical Compounds: 1,1-diohenyl-2-picryl-hydrazil (PubChem CID: 2735032); 15 Ethanol (PubChem CID: 702); Methanol (PubChem CID: 887); Cloridric Acid 16 (PubChem CID: 2797); Sodium hypochlorite (PubChem CID: 23665760

1. Introduction

Natural antioxidants present in foods and other biological materials have attracted considerable interest because of their presumed safety and potential nutritional and therapeutic value. The increased interest in natural antioxidants has led to the antioxidant evaluation of many species of fruits, vegetables, herbs, spices and cereals (Rufino *et al*., 2010; Velioglu *et al*., 1998; Wolfe *et al*., 2003). In addition, the number of studies on residual sources of antioxidants has increased considerably in recent years (Babbar *et al*., 2011; Moure *et al*., 2001).

Since the disposal of these by-products still is problematic in Brazil, due to the fact that mostly is badly discarded causing environmental problem, and increasing economic losses (Melo *et al*., 2011), the production of nutraceuticals using bioactive compounds from fruit residues will help in efficient, inexpensive, and environmentally friendly use of these fruit residues. So, one of the most effective options is the recovery of the bioactive constituents, which could be used in food, cosmetic, and pharmaceutical industry (Makris *et al*., 2007).

Antioxidant compounds from agri-waste may not only increase the stability of foods, by preventing lipid peroxidation, but in humans or animals may also protect biomolecules from oxidative damage (Ayala-Zavala *et al*., 2011; Babbar *et al*., 2011).

* Corresponding author
E-mail: ediracba@analisedealimentos.com (E. Gonçalves).

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It is well known that the antioxidant capacity of food is derived from the synergistic action of a wide variety of antioxidants. For that reason, it is usually necessary to combine more than one method in order to determine in vitro, the antioxidant capacity of food stuffs (Frankel and Meyer, 2000; Pérez-Jiménez *et al*., 2008). However, it is also important to note that even within a method a small difference in solvent polarity may provide different responses (Turkmen *et al*., 2006).

These antioxidants solubility in a specific solvent is a peculiar characteristic of the phytochemicals present in the food matrix, which explains the inexistence of a universal procedure to measure the total antioxidant capacity and phenolic content. It also demonstrates the need for a meticulous selection of the extraction method for each natural source of antioxidants (Caetano *et al*., 2011; Frankel and Meyer, 2000; Sanchez-Moreno, 2002).

Solvents extraction using water, methanol, ethanol, acetone, propanol, ethyl acetate, and dimethylformamide in various proportions have been commonly used for the extraction of phenolic from fruits and their waste. This is due to wide range of phenolic compounds that these extractants can dissolve (Caetano *et al*., 2011). Still, the extraction method must enable complete extraction of the compounds of interest and must avoid their chemical modification (Zuo, 2002).

According to Pérez-Jiménez and Saura-Calixto (2006) the type of solvent and polarity may affect the single electron transfer and the hydrogen atom transfer, which are key aspects in the measurement of antioxidant capacity. In addition, aqueous-organic extracts may contain not only the antioxidants but also other non-antioxidant food constituents that may interfere in antioxidant capacity assays.

The mechanisms of sequential extraction in foods, using different extractors are very commonly used, especially because this extraction method can provide a better understanding of bioactive compounds and their bioavailability, since it considers the variability of the phytochemicals present on each sample and the influence of solvents type in such systems (Litwinienko and Ingold, 2005, 2004, 2003).

Therefore, was recently proposed and elaborated an isotonic beverage manufacture based on integral exploitation of several fruits and vegetables species (Martins *et al.*, 2011). According to methodology and characterization described by Ferreira *et al.* (2015), this beverage solid residue was processed into flour (FVR). So that, the aim of this research was to investigate the effect of extracting solvents with different proportion of methanol, ethanol and water, on antioxidant activity and phenolic compounds of a fruit and vegetable residue flour (FVR), by DPPH and Folin-Ciocalteau methods, and these compounds stability during storage.

2. Material and methods

2.1. Sample preparation

A pre-defined amount of the selected fruits and vegetables were purchased from a local supermarket (Rio de Janeiro, Brazil) and processed following a method described by Ferreira *et al.* (2015) previously: Selecta orange (*Citrus sinensis*), passion fruit (*Passiflora edulis*) and watermelon (*Citrullus lanatus*), lettuce (*Lactuca sativa*), courgette (*Cucúrbita pepo*), carrot (*Daucus carota*), spinach (*Spinacea oleracea*), mint (*Mentha* s.p.), taro (*Colocasia esculenta*), cucumber (*Cucumis sativus*) and rocket (*Eruca sativa*).

All fruits and vegetables were properly washed in flowing water. They were sanitized for 30 min in a bath containing 200 ppm of sodium hypochlorite (NaClO) before rinsing in flowing destilated water. After the production of a concentrated juice, the remaining solid residue (FVR) was immediately processed into flour.

The FVR was dried in a drying oven with air renewal and circulation (Marconi, model MA035, Brazil) at 65 °C for 6 h. Then, the dehydrated residue was ground using a food processor for 5 min and dried

out for 1 h at 90 °C before grinding once more for 1 min (Ferreira *et al*., 2013). Three flour samples were prepared at intervals of 15 days between them. All samples were stored at room temperature in aluminized aseptic bags until further analysis.

To evaluate FVR bioactive compounds stability, all analysis were performed considering 0, 30, 60, 90, 120 and 180 days of storage.

2.2. Extraction

For each extract, 10 mg of dried sample was extracted with 10 ml of ethanol (E), ethanol/methanol (I) 50:25 (v/v), (II) 50:50 (v/v), (III) 25:50 (v/v), methanol (M) and water (W), at room temperature for 60 min. Extractors were applied separately and sequentially. After the extraction process, all extracts were centrifuged during 10 min, at 2000 x g, then they were separated from de solid part and only the supernatant was used. Three extraction replicates were prepared for each sample of FVR along storage.

2.3. Free Radical Scavenging Activity assay by DPPH

The free radical scavenging activity of the extracts were measured in terms of hydrogen-donating or radical scavenging ability, using 1.1-diphenyl-2-picryl-hydrazil (DPPH) (Brand-Williams et al., 1995) with few modifications.

A 60 µM solution of DPPH was prepared and 2.0 ml of this solution was added to different volumes of FVR extracts at room temperature for 60 min, to ensure the development of the reaction. The mixture was kept in the dark prior to analysis, and then the absorbance was read at 517 nm, using spectrophotometer (Shimadzu, UV-2700). Blank samples were prepared where DPPH was replaced with methanol. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The radical scavenging activity (RSA) was calculated as a percentage of DPPH discoloration using the equation: % RSA = $[(A_{DPPH} - A_{EXT})/A_{DPPH}]$ x 100. Were A_{DPPH} is the absorbance value of the DPPH sample, and A_{EXT} is the absorbance value of the test solution. A_{EXT} was calculated as the difference between the absorbance value of the test solution and the absorbance value of its blank.

2.4. Phenolic Compounds assay

Total phenolic content of FVR extracts was assessed using a modified version of the Folin–Ciocalteu assay (Singleton et al., 1999). The concentrated alcoholic extracts rates were oxidized with Folin-Ciocalteu's reagent (2.5 ml) and then neutralized with saturated sodium carbonate solution (2.0 ml). Samples were adjusted to 5.5 ml, with the specific extractor used.

The final volume (5.5 ml) was allowed to stand for 120 min at room temperature, then the absorbance was measured at 750 nm using a spectrophotometer (Shimadzu, UV-2700). An aqueous Gallic acid solution (0.1 mg L^{-1}) was used as a standard to prepare a calibration curve, and the content of phenolic in each extract was calculated from the regression equation of gallic acid calibration curve and expressed as milligrams of gallic acid equivalent (GAE) per 1 gram of sample.

2.5. FVR flour microstructure

Structure (shape and size) of FVR flour were analyzed by using Scanning Electron Microscope (SEM) (Oxford Industries, England) coupled with X-ray Energy Dispersive Spectrometer (EDS) (Oxford Industries, England). Samples were put on aluminum holders and covered with a gold layer (40-50 nm) for 100 seconds. For SEM analysis, each sample was viewed at an acceleration voltage in the range of 15 – 20 kV and EDS signal was recorded at 20 kV acceleration voltage. Analyses were performed in six random sample points.

2.6. Statistical Analysis

All data were found to be normally distributed using the Kolmogorov-Smirnov test. The one-way ANOVA analysis of variance followed by Tukey's test were also applied, and results were considered

statistically significant with a 95% confidence level ($p < 0.05$). All results were examined using the software Assistat (7.6 beta, 2013).

3. Results and discussion

FVR extracts clearly displays a dose-dependent antioxidant activity against DPPH radical in all extracts (Figure 1), however, this reaction appears to be limited, which was noted by the non-proportional raise of reduction percentual, that should obey Lambert-Beer law (Blois, 1958). This may be caused by the reaction between DPPH and the solvents (Arnao, 2000), since it seems to be more problematic with the methanol:ethanol mix extracts.

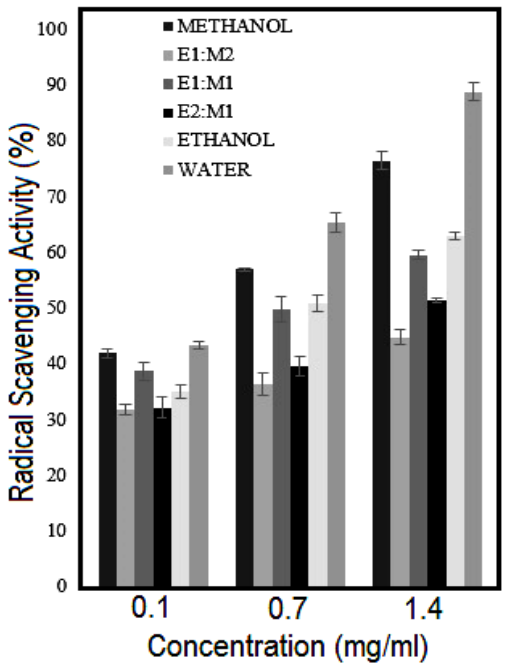

Figure 1. Radical Scavenging Activity of FVR, with non-sequential extraction, using six different extractants (n=3).

It was also possible to observe, in non-sequential extraction, results ranging from 44.85% to 88.92% in E/M (25:50 v/v) and water respectively, on the rates of 1.4 mg/ml. Aqueous extract presented more promising results, followed by the higher polarity solvents methanol (100%) and ethanol (100%) that range 76.52% and 63.06% respectively.

As neither the concentration nor the mixture of bioactive compounds and solvent present in 1.4 mg/ml aliquots appeared to serve as an interfering to the reaction in the non-sequential assays, a sequential trial began based on the polarity of the extractants used. A first sequence was determined starting with the most polar (water) going to the most nonpolar (ethanol).

The results in Figure 2 shown that starting the fourth puller E/M (50:50 v/v) occurred a loss of linearity unexpected for this type of analysis, however even then it is noted that the extraction is done effectively, causing a reduction rate of 82.98% water and 34.32% ethanol.

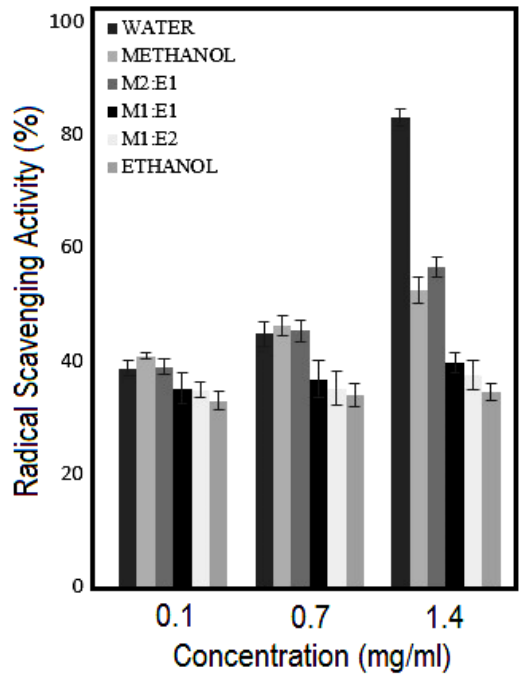

Figure 2. Radical scavenging activity of FVR with water-ethanol sequential extraction (n=3).

The similarity between the values found in non-sequential and sequential extractions using water as solvent is also observed in Figure 2. It also shows that with the addition of extractors sequentially, it is possible that some reaction has occurred with the solvents mixture or with the sample-solvent mixture, capable of interfe-

ring with its absorbance, starting with the fourth puller. These results demonstrate that extraction yield is dependent on the solvent and method of extraction as observed by (Goli et al., 2005).

Pérez-Jiménez and Saura-Calixto (2006) observed that when compared to other antioxidant activity assays, DPPH is the method in which the solvent influence is weakest when simple constituents such as different glucids, nitrogenated amino acid and common protein were evaluated. They also recognized that this influence might be greater if the sample analyzed were food – a complex matrix in which different compounds may establish different interactions between them and the solvents.

When inverted the order of extractors, beginning with ethanol (nonpolar) and ending with water (polar), it was observed a maintenance of results linearity, which increases data reliability when compared with previous sequence analysis. However, percentage reductions were significantly lower, ranging between 53.45% and 47% in the first and last extract respectively, as shown in Figure 3. It demonstrate how important it is to establish, an ideal sequence of pullers, once their different polarity, or even a slice mixture of extract, or an interaction between the solvent and food constituents can represent an important interference to DPPH radical reaction (Pérez-Jiménez and Saura-Calixto, 2006).

Based on previous results FVR storage assays started using a sequential extraction following ethanol-water sequence. As results to these tests, the antioxidant capacity decreased significantly during storage of FVR, ranging from 53% to 39% in the first extractor (ethanol), between 0 and 180 days and from 50% to 44% on the last extractor at the same time (Figure 4). Although a significant reduction of antioxidant capacity of FVR was observed, it seems to be quite unimportant since the activity remains notable after 180 days, which indicates the possibility of storage FVR for long periods.

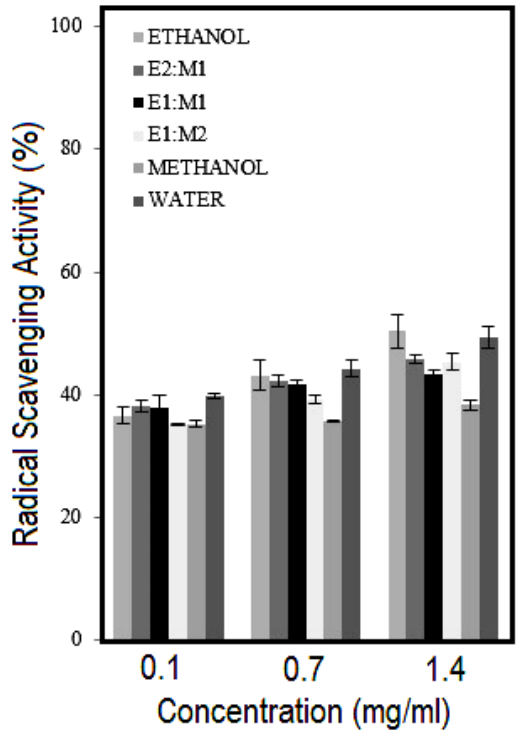

Figure 3. Radical scavenging activity of FVR with ethanol-water sequential extraction (n=3).

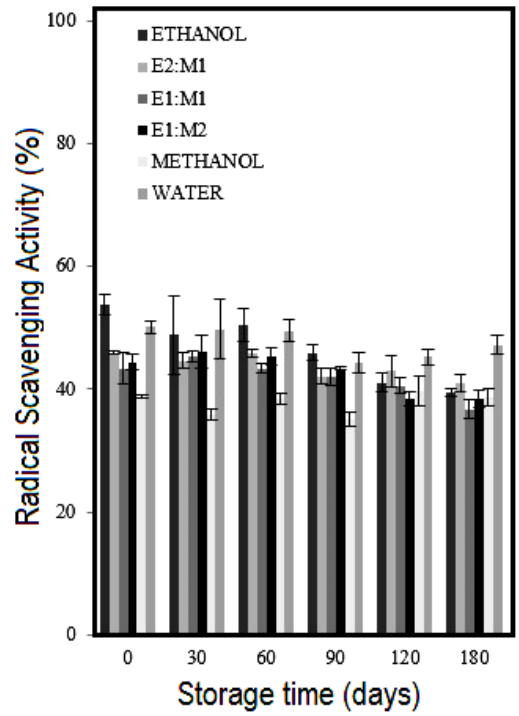

Figure 4. Radical scavenging activity of FVR, with ethanol-water sequential extraction during 180 days (n=3).

To phenolic compounds, it was observed that ethanol was the most efficient extractor. On the first day of analysis 22.49 ± 1.59 mg GAE/g were obtained from FVR. During storage that amount presented a reduction of approximately 77% after 180 days. It was also noted different extraction profile for phenolic compounds when compared to antioxidant capacity assay. There were significant changes on the extraction profile when phenolic compounds were evaluated, along storage time, characterized by a non-extraction of bioactive compounds observed from day 90 in third extractor (Figure 5).

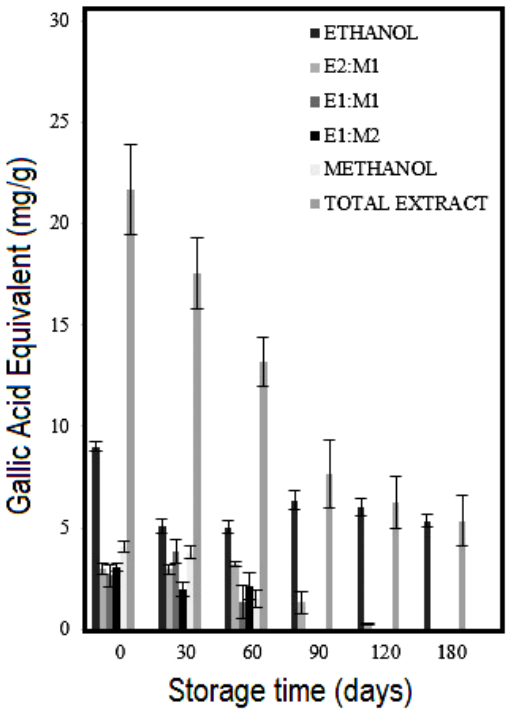

Figure 5. Phenolic compounds with sequential extraction, free and bound phenolic compounds of FVR during 180 days of storage (n=3).

Lower values were found by Sun et al. (2002) to apple (2.96 ± 0.064mg/g GAE), banana (9.90 ± 0.032 mg/g GAE), pineapple (0.94 ± 0.015 mg/g GAE), cramberry (5.27 ± 0.21 mg/g GAE), peach (0.85 ± 0.07 mg/g GAE) and strawberry (1.6 ± 0.12 mg/g GAE). Also lower values were found on oven-dried at 60°C apple peels samples analyzed by (Wolfe and Liu, 2003) as 3.03 mg/g fresh peels GAE.

Particles of different sizes in flours characterize the molecular matrix degradation (Roman-Gutierrez et al., 2002). In figure 6, chemical structure degradation is verified after storage.

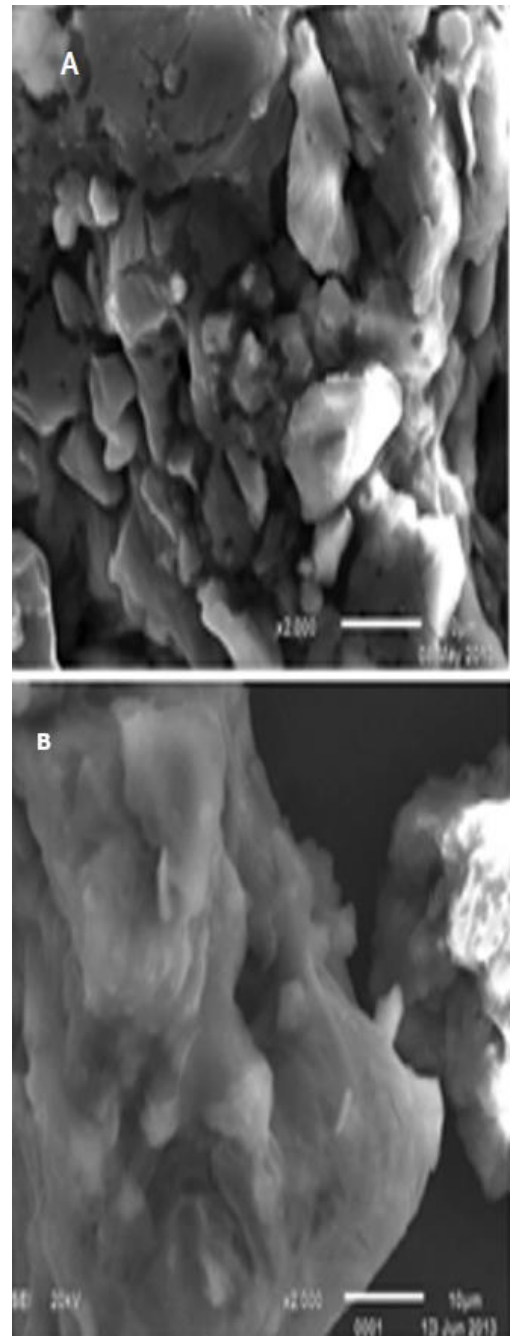

Figure 6. Scanning electron micrographs (SEM) of fruit and vegetable residue flour (FVR): (A) control; (B) after storage.

This influence is mainly observed on phenolic compounds degradation. When evaluated the antioxidant capacity of FVR samples, according to DPPH radical reducing process, the noted matrix degradation during storage time does not influence significantly antioxidant activity, but these might be conserved by bioactive compounds naturally present on the samples or even by different compounds that might be formed from degradation process during storage time. Similar results were observed by (Rotili *et al*., 2013) where an increasing on total phenolic compound of yellow passion fruit during storage time under different temperatures occurred.

4. Conclusions

By-products phenolic compounds are present in different binding status depending on plant species, therefore extracting solvent significantly affected antioxidant activity of fruits and vegetables residue flour (FVR). Ranking in the antioxidant activity of extracts varied depending on the polarity of solvent and the method used to extract bioactive compounds. However, regardless of the method used, the most efficient solvents for antioxidant activity were 100% water, and ethanol. In non-sequential extraction, polyphenol content of E/M (50:25 v/v) extract was the lowest. When in water-ethanol sequential extraction it was E:M (50:50 v/v), and to ethanol-water was 100% methanol. As to phenolic compounds, ethanol was also the best extractor to all evaluated samples.

All samples demonstrate to remain its antioxidant capacity, and phenolic compounds during 180 days. That can be explained by several transformations that occurs on samples during storage that changes the matrix structure allowing different interactions between FVR compounds.

It is also noted that both analyses demonstrate losses occurring along storage time. When compared to other by-products or fresh fruits, FVR showed to have a higher antioxidant capacity and phenolic compounds, than some samples, which demonstrates the importance of using industrial residue in order to contribute to a better utilization of all residue generated, reducing costs and environmental impact, and developing ways to apply those residue sources to improve human health.

For a better understanding of the mechanism involving the potential antioxidants of FVR, it would be interesting to identify and characterize these compounds.

Acknowledgements

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