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FOOD TECHNOLOGY

# Cytotoxicity and antioxidant activity of goldenberry extracts obtained with high intensity ultrasound

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**ABSTRACT**: *The high intensity ultrasound-assisted extraction (HIU) is one of the most simple, quick and efficient techniques for the extraction of phenolic and other antioxidant compounds from plants. This is the first application of HIU for the extraction of these compounds from goldenberry fruit. The HIU and conventional extraction techniques showed similar results regarding to phenolic compounds and antioxidant capacity. However, the time required for HIU extraction (5min) was 24 times lower than conventional extraction (120min). Phenolic compounds reported were chlorogenic acid, caffeic acid and rutin. In vitro cytotoxicity assays were used for evaluation of extracts and the results showed that in a wide range of concentration, the extract maintains cell viability, thus indicating the possibility to use it as food with safety.*
**Key words**: *goldenberry; sonication; antioxidant capacity; cytotoxicity; high performance liquid chromatography.*

## Citotoxicidade e atividade antioxidante de extratos de goldenberry obtidos com ultrassom de alta intensidade

**RESUMO**: *A extração assistida com ultrassom de alta intensidade (HIU) é uma das técnicas mais simples, rápidas e eficientes na extração de compostos fenólicos e antioxidantes de plantas. Este trabalho foi o primeiro a utilizar HIU na extração destes compostos presentes na fruta goldenberry. As técnicas HIU e extração convencional apresentaram resultados semelhantes com relação aos compostos fenólicos e capacidade antioxidante. Entretanto, o tempo necessário na HIU (5min) foi 24 vezes menor que na extração convencional (120min). Os compostos fenólicos encontrados foram ácido clorogênico, ácido cafeico e rutina. Ensaios de citotoxicidade in vitro foram usados para avaliação dos extratos e os resultados demonstraram que, em ampla faixa de concentração, o extrato mantém a viabilidade celular, indicando assim possível segurança para utilização em alimentos.*
**Palavras-chave**: *goldenberry, sonicação, capacidade antioxidante, citotoxicidade, cromatografia líquida de alta eficiência.*

## INTRODUCTION

Goldenberry (*Physalis peruviana* L.) belongs to the family Solanaceae, which have about 120 species, distributed in tropical and subtropical regions of Africa, Asia and America (KUSPKA & JÉLEN, 2016). The bioactive compounds present in the fruit of goldenberry, especially phenolic compounds, have important pharmacological properties, such as antioxidant, antibacterial, antitumor, antinflammatory and immunosuppressive action (KUSPKA & JÉLEN, 2016).

The extraction is the first step to obtain bioactive compounds from plant materials, and conventional solvent extractions, such as maceration, percolation, soxhlet and stirring methods, are the most widely used (PATIL & AKAMANCHI, 2017). However, these methods consume large amounts of solvents with poor extraction efficiency, long times of extraction with low efficiency (SHIRSATH et al., 2012). As an innovative technology, HIU has been proposed in recent years as a tool to improve extraction efficiency and reduce solvent consumption (CHEMAT et al., 2013; KUMARI et al., 2017).

For goldenberry, the extraction of phenolic compounds with ultrasound was performed only by the use of baths (LICODIEDOFF et al., 2013) and no applications of HIU were reported. In addition, the information about the toxicity of extracts of goldenberry is scarce. Thus, in

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the present research, HIU was compared with conventional solvent extraction by using the determination of total phenolic compounds and antioxidant capacity method ORAC (Oxygen Radical Absorbance Capacity). A high performance liquid chromatography (HPLC) method was developed for the chromatographic separation, identification and simultaneous quantitative determination of phenolic compound in each extract. The in vitro cytotoxicity of the obtained extracts using non-tumor cell lines (3T3, murine Swiss albino fibroblasts) and two cytotoxicity assays, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and neutral red uptake (NRU) assay were also performed.

## MATERIALS AND METHODS

*Plant material and reagents*

Goldenberry were purchased from Italbraz (Vacaria, Brazil) located at 28:0:44 S and 50:56:02 W. Samples were stored at -18°C till analyses. All extract were prepared with peel, pulp and seeds of the ripe fruit. All reagents used were from analytical grade. Gallic acid, Trolox, AAPH, fluorescein, caffeic acid, chlorogenic acid, rutin, phosphate buffered saline (PBS), MTT, NR dye, DMSO and trypsin-EDTA solution were obtained from Sigma-Aldrich (St. Louis, USA). Folin-Ciocalteau reagent and formic acid were obtained from Merck (Darmstadt, Germany), sodium carbonate was purchased from Vetec (Duque de Caxias, Brazil); Fetal bovine serum (FBS) and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Vitrocell (Campinas, Brazil).

*Conventional extraction method*

Extracts were obtained following the method used by ROCKENBACH et al. (2008) with some modifications. Fresh fruit samples (15g) were ground and 25mL of extraction solvent (methanol or ethanol 60% (v/v)) was added with magnetic stirring protected from light for two hours. The extracts were filtered through a 0.45µm polyamide filter (Sartorius Stedim Biotec) and stored until analysis.

*High intensity ultrasound-assisted extraction*

For HIU, an ultrasound probe (130W, 20kHz, Sonics and Materials Inc., USA) was used. Samples of fresh fruits (15g) were ground and placed in a stainless steel water-cooled reactor, at 4.0°C. Then, 25mL of solvent (methanol or ethanol 60% (v/v)) was added with further sonication by 5 or 15min using a power output of 70%. Extracts were filtered through quantitative filter paper and stored until analysis.

*Determination of total phenolic compounds*

The determination of total phenolic compounds was performed by spectrophotometric Folin-Ciocalteu method (CHANDRA & MEJIA, 2004). Gallic acid was used as reference solution for calibration curve from 0.001 to 0.03mg/mL. Results were expressed as milligrams of gallic acid equivalent per 100 gram of fresh fruit (mg GAE/100g).

*HPLC analysis*

Analyses were performed using Luna RP-18 column (250x4.6mm, 5µm, Phenomenex, USA) and an Agilent 1260 Infinity (Agilent Technologies, Germany) equipped with G1314B UV detector, G1311C quaternary gradient pump, and G1329B autosampler. The mobile phase consisted of a gradient mixture of solvent A (0.45% formic acid in Milli-Q water) and B (methanol). The gradient was as follows: 20% B (5min), 20% to 52% B (6min), 52% B (17min), and then returns to initial conditions by 3min. The flow rate was 0.8mL/min, and the injection volume was 20µL. Phenolic compounds were monitored at 370nm. Extracts and standard compounds were analyzed under the same analysis conditions.

*Antioxidant capacity evaluation*

The ORAC assay was performed as described by OU et al. (2001) by using 96-well microplates (SPL Life Science, Gyeonggi-do, Korea). This kinetic assay is based on the measurement of radical scavenging activity of extract against peroxyl radicals produced by the addition of AAPH radical inductor. Antioxidant capacity was determined using area under curve (AUC) and results were compared to a standard curve of Trolox (from 8 to 96µmol/L) and expressed as Trolox equivalents of µmol/gram of fresh fruit.

*Cytotoxicity assays*

The 3T3 cells were seeded into 96-well plate at a density of 1x $0^5$cells/mL. After incubation for 24 h under 5% $CO_2$ at 37ºC, the spent medium was replaced with 100µL of fresh medium containing the extract at the concentration range 15.6-2000µg/mL or with 100µL of medium only for the untreated control cells. After 24h, the extract-containing medium was removed, and 100µL of MTT (0.5mg/mL) or NR (50µg/mL) solution in FBS-free medium was added for the MTT and NRU assays, respectively. Plates were further incubated for 3h, after which the medium was removed. Afterward, 100µL of DMSO or of a solution containing 50% absolute ethanol and 1% acetic acid in distilled water was then added. Absorbance of solutions was measured at 550nm

using SpectraMax M2 microplate reader (Molecular Devices, CA, USA). Cytotoxicity of the extract was expressed as percentage of viability with regard to untreated control cells (the mean optical density of untreated cells was set at 100% viability).

*Statistical analysis*

Statistical analysis of results were performed using analysis of variance (ANOVA) and Tukey test ($p<0.05$). Results were expressed as mean ± standard deviation of three independent experiments. The cytotoxicity was expressed as percent of viability with regard to untreated control wells, using mean ± standard deviation of two independent experiments.

## RESULTS AND DISCUSSION

In order to study the best method of extraction, the determination of total phenolic content and antioxidant activity of extracts was determined. In this way, conventional and HIU extractions were performed with two different solvents. As shown in table 1, no significant difference ($p>0.05$) was observed for different extraction techniques as well as for different solvents regarding to the amount of total phenolic compounds. Contents of total phenolic compounds reported in this study (around 610mg GAE/100g of fresh fruit) were higher than those found by ROCKENBACH et al. (2008) and LIMA et al. (2012), which were 57.9mg GAE/100g of fresh fruit in methanolic extract and 187.59mg GAE/100g of fresh fruit in aqueous extract, respectively. The selection of the most appropriate solvent for extracting the compounds from the matrix is one of the most important operational parameters in the extraction methods (NGO et al., 2017).

Quantitative analysis of the phenolic compounds reported in goldenberry extracts was performed using HPLC. The following phenolic compounds were identified in the extracts: chlorogenic acid, caffeic acid and rutin (Table 2), and the retention time were 10.8, 11.5 and 14 minutes (Figure 1), respectively. Chlorogenic acid and rutin have been reported in the goldenberry in other studies (LICODIEDOFF et al., 2013; ROCKENBACH et al., 2008). Together with caffeic acid, these phenolic compounds are responsible for a number of biological activities including antioxidant.

Table 1 - Content of total phenolic compounds after conventional and high intensity ultrasound-assisted extraction.

| Method | mg GAE/100g of fresh fruit ± SD | |
|---|---|---|
| | 60% Ethanol | 60% Methanol |
| Conventional extraction | 540±60 | 542±30 |
| HIU 5min | 612±87 | 608±70 |
| HIU 15min | 581±43 | 598±16 |

In this way, the antioxidant capacities of these extracts were evaluated. The table 3 presents the results for antioxidant activity of extracts obtained by use of conventional and HIU extraction. Statistical analysis (ANOVA) indicated that the extractions with ethanol and methanol were different. The antioxidant capacity of extract obtained with ethanol was higher. DO et al. (2014) investigated the effects of solvents in the extraction of phenols and antioxidant capacity from *Limnophila*. They observed that the extraction with ethanol and acetone showed no significant difference in the content of phenolic compounds. However, the antioxidant capacity, measured by different methods, showed that the extracts obtained using ethanol presented statistically higher values than acetona. The authors concluded that the different solvents used in extraction resulted in differences in compositions and antioxidant capacities of the extracts. PANIWNYK et al. (2009) evaluated the effect of different solvents in the extraction of antioxidant compounds from rosemary (rosmarinic acid and carnosic acid), using ultrasonic and conventional extraction. The authors observed that methanol was a more effective solvent for the extraction of total antioxidants than ethanol. However, the analysis of the extracted antioxidant compounds showed that methanol was a better solvent for rosmarinic acid, while ethanol was a better solvent for carnosic acid. Therefore, depending on the solvent used for extraction, different amounts of antioxidant compounds can be extracted.

In the literature, there are different methods for evaluation of the antioxidant capacity of goldenberry, including the trolox equivalent antioxidant capacity (TEAC), 2,2'-diphenylpicryl hydrazyl free radical (DPPH), thiobarbituric acid reactive substances (TBARS), 2,2'-azobis (2-amidinopropane) dihydrochloride (ABTS), ORAC, among others (KUSPKA & JÉLEN, 2016; LICODIEDOFF et al., 2013; ROCKENBACH et al., 2008). The ORAC is the most widely recognized of all of these antioxidant assays. The method uses the peroxyl radical as pro-oxidant, which is the best model of antioxidant reaction with reactive oxygen species in foods and in vivo (NGO et al., 2017). Moreover, the reactive species interact with the

Table 2 - Phenolic compounds in goldenberry extracts.

| Method | Solvent | Chlorogenicacid $T_R^*$ (min) 10.8 | Caffeic acid $T_R^*$ (min) 11.5 | Rutin $T_R^*$ (min) 14.0 |
|---|---|---|---|---|
| | | Concentration (μg/g of fresh fruit) ± SD | | |
| Conventional extraction | Ethanol 60% | 42.3±3.7 | 9.8±1.3 | 4.1±0.2 |
| HIU 5min | | 22.6±3,6 | 6.7±1.8 | 5.1±0.4 |
| HIU 15min | | 20.0±2.4 | 5.8±1.6 | 5.1±1.8 |
| Conventional extraction | Methanol 60% | 36.3±1.4 | 7.3±2.9 | 4.5±0.8 |
| HIU 5min | | 18.5±2.1 | 5.7±1.5 | 4.2±0.1 |
| HIU 15min | | 27.7±8.0 | 5.9±1.5 | 4.1±0.3 |

$T_R^*$: Retention time

substrate until the end of assay and the area under the curve is used for quantitation, while the other methods use a percentage of inhibition at a fixed time (SEPTEMBRE-MALATERRE et al., 2016).

Results of determination of total phenolic compounds and antioxidant capacity did not demonstrate significant difference among the different extraction techniques. However, the time of extraction for conventional method was 120min, while HIU was only 5min, which is an important advantage. The time required for extraction will typically depend on the type of material, the cell wall structure, mass transfer resistance for the diffusion of the solvent into the material and the penetration rate of the solvent to plant material (SHIRSATH et al., 2012).

Therefore, the best extraction method was HIU using ethanol as extraction solvent. It is important to mention that ethanol is less toxic than methanol, being generally recognized as a safe solvent being more suitable for further application of

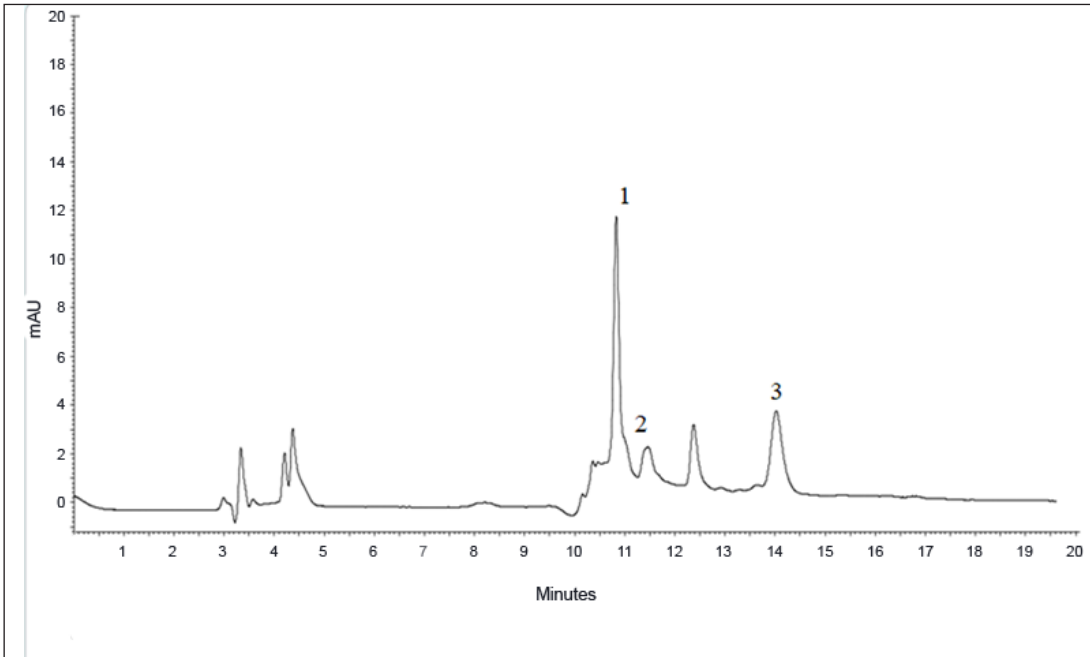


Figure 1 - Chromatogram of the phenolic compounds from ethanolic extract of goldenberry: chlorogenic acid (1), caffeic acid (2) and rutin (3).

Table 3 - Antioxidant capacity of goldenberry extracts by different extraction methods using different solvents.

| Method | µmol trolox equivalent/g of fresh fruit ± SD | |
|---|---|---|
| | 60% Ethanol | 60% Methanol |
| Conventional extraction | 1067±256[a] | 456±131 [b] |
| HIU 5min | 1251±127[a] | 354[b]±25 [b] |
| HIU 15min | 1212±82[a] | 337[b]±46[b] |

Mean ± standard deviation followed by the same letters in the same column, indicate statistically similar at the 0.05 level.

goldenberry extract in food (KUMARI et al., 2017). In order to evaluate the cytotoxicity potential of the ethanol extract of goldenberry two in vitro bioassays, MTT and NRU, were compared.

Cytotoxicity evaluated with non-tumor cell line is an important tool for the prediction of risk and safety assessment of the potential toxicity of bioactive compounds. *In vitro* systems are mainly used for screening purposes and for generating more comprehensive toxicological profiles (EISENBRAND et al., 2002). It was observed in the NRU assay that a 24h-exposure of cell line to different concentrations of ethanolic extract caused no reduction in the number of viable cells, whereas in the MTT assay there was a slight reduction in cell viability at the concentration of 2000mg/mL (Figure 2). Apparently this reduction did not represent cytotoxicity, since the values were above 75% (ISO 10993-5) (WANG et al., 2015).

## CONCLUSION

This was the first study using high intensity ultrasound-assisted extraction focused on the phenolic and antioxidant compounds from fruits of goldenberry. In comparison with conventional extraction, HIU reduced the time of extraction 24 times with the same efficiency. Ethanol was a better extraction solvent than methanol. Examination of the extracts revealed the presence of phenolic compounds important for human health, like chlorogenic and caffeic acid, and rutin. This research also conducted two *in vitro* cytotoxicity assays, MTT and NRU, and it was observed that over a wide range of concentration, ethanolic extract of goldenberry did not show *in vitro* cytotoxicity, demonstrating the possibility of use in food.

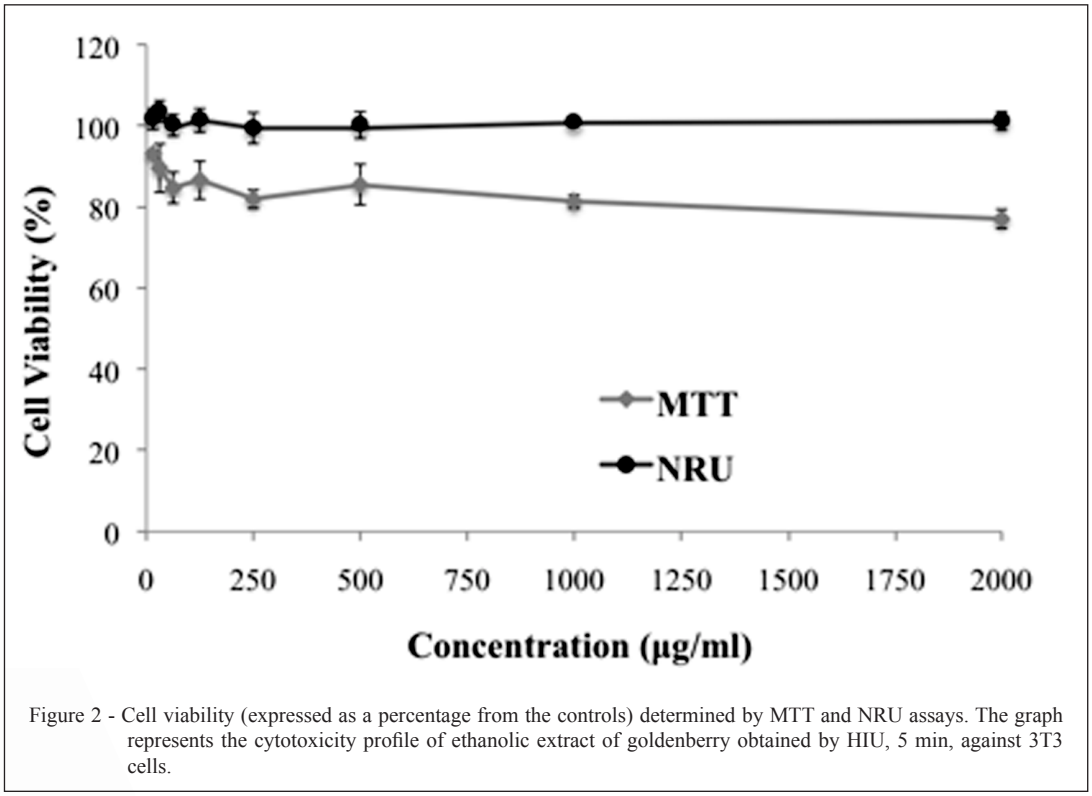


Figure 2 - Cell viability (expressed as a percentage from the controls) determined by MTT and NRU assays. The graph represents the cytotoxicity profile of ethanolic extract of goldenberry obtained by HIU, 5 min, against 3T3 cells.

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