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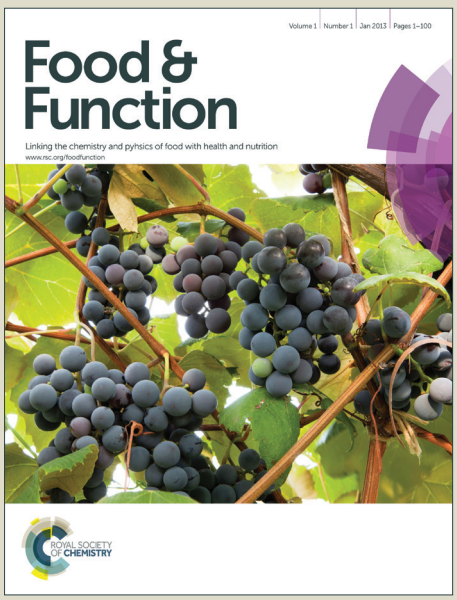

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Piceid Presents Antiproliferative Effect on Intestinal Epithelial Caco-2 Cells, Effects Non Related with Resveratrol Release

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Abstract*: Trans*-Piceid (T-Pc) is abundant in Polygonum cuspidatum and in grape and grape products such as wine. Piceid has a rapid oral absorption reaching high levels in stomach and intestine. Tissues such as liver can deglycosylate piceid to release resveratrol, considering piceid as a reservoir of resveratrol that has numerous biological activities such as antiproliferative effects. Thereby, the aim of this work was analyze the action of T-Pc on intestinal epithelial cell growth. Our results show that T-Pc has antioxidant activity similar to that *trans*-resveratrol (T-Rv) and higher than Trolox activity. Moreover, T-Pc (1-50 μM) inhibited Caco-2 cell growth and DNA synthesis in a concentration-dependent manner. We observed an increase in the percentage of cells in G_0/G_1 phase induced by T-Pc and the induction of apoptosis. Furthermore, we observed that Caco-2 cells did not have β-glucosidase activity and that Caco-2 cell cultures did not deglycosylate significantly T-Pc in our experimental conditions. On the basis of our results we propose, for the first time, that T-Pc must not be considered exclusively as T-Rv reservoir and presents antiproliferative effect on intestinal epithelial cells through the modulation of cell cycle and apoptosis by itself. Moreover, a synergistic action of T-Pc and T-Rv can be considered.

Keywords: Polyphenol, resveratrol, cell growth, apoptosis, colorectal cancer

Introduction

Piceid (*trans*-resveratrol-3-*O*-β-glucoside), also named polydatin, is the main metabolite/precursor of resveratrol in plants. It is more abundant in *Polygonum cuspidatum*, a weed that is used in traditional Chinese and Japanese medicines. The presence of *trans*-resveratrol (T-Rv), *trans*-piceid (T-Pc) and their respective *cis*-isomers in the human diet are limited. They are principally found in grapes and grape derivatives such as grape juices and wines,[1-4] where the level of the T-Pc in red wine may be 10 times greater than the level of T-Rv.[5-7] Although there are other sources like peanuts,[8] pistachios[9] and some berries,[10] their total resveratrol levels are from 10 to 100-times lower than those in grape products.[11] Recently, Zamora-Ros et al.[4] reported that T-Rv, and especially T-Pc, are common components of the Mediterranean diet and wine was found to be the main source with an estimated consumption mean of T-Pc of 500 μg/day.

It is well known the numerous biological effects of resveratrol[12] but there is little information about the biological activity of T-Pc, the predominant form of T-Rv in foods. The aglicone T-Rv seems to have a greater biological effect than the T-Pc.[13,14] However, it has been found that T-Pc can inhibit platelet aggregation, improve circulation, recover tissue damage induced by ischemia reperfusion, lower blood cholesterol and suppress lipid peroxide formation.[15,16] T-Rv plays numerous biological roles, possessing anti-inflammatory[17] and antiproliferative/chemopreventive[18] activities, but the action and mechanisms of antiproliferative effect of T-Pc on human tumor cells remain vague.

Actually, there are some references that supports the hypothesis that deglycosylation by the β-glycosidases of the intestine is the first step in the absorption and metabolism of dietary polyphenol glycosides.[19-22] Thus, the deglycosylation of T-Pc could increase the levels of T-Rv available from diet.[20,23] However, we can not exclude that T-Pc may be active on intestinal epithelium. Considering all together, the aim of

this study was to clarify the action of T-Pc on intestinal epithelial cell growth and propose a mechanism to its biological effects.

Experimental

Materials

Dulbecco's modified Eagle's medium (DMEM), trypsin/EDTA, penicillin, and streptomycin were supplied by GIBCO. Nonessential amino acids, fetal bovine serum (FBS), Dulbecco's PBS, propidium iodide, Triton X-100, ribonuclease A from bovine pancreas, bovine serum albumin (BSA), acridine orange, ethidium bromide, potassium ferricyanide, ferric chloride, gluconolactone, α-glucosidase, β-glucosidase, 4-nitrophenyl-α-D-glucopyranoside, 4-nitrophenyl-β-D-glucopyranoside and quercetin were supplied by Sigma-Aldrich. Tissue culture supplies and sterile material were obtained from Corning, Nirco S.L., NORM-JECT and Biosigma S.R.L. [Methyl-^{3}H] thymidine (20 Ci/mmol) were supplied by American Radiolabeled Chemicals Inc. Paraformaldehyde extra pure was from Merck Chemicals. The BioRad Protein Assay was obtained from Bio-Rad Laboratories, Inc. T-Rv and T-Pc was supplied by Cayman Chemical.

Cell and Bacterial Culture

Caco-2 cells were derived from a moderately well-differentiated primary colon adenocarcinoma and were provided by American Type Culture Collection (HTB-37) (Manassas, VA, USA). The cells (passages 19-40) were routinely grown in 25 or 75 cm^2 plastic flasks at a density of 2-2.5 x 10^4 cells/cm^2 and cultured in DMEM with 4.5 g/L D-glucose and 2 mM L-glutamine, and supplemented with 1% (v/v) nonessential amino acids, 10 % (v/v) heat-inactivated FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were incubated at 37 ºC under a humidified atmosphere of 5 % CO_2 in air. Cells grown to ~80 % confluence were released by trypsinization and

subcultured at a density of 1.5-2 x 10^4 cells/cm^2 in 12 mm diameter plastic clusters and of 1.5 x 10^4 cells/cm^2 in 60 mm diameter plastic dishes. Growth medium was replaced twice per week. The experiments were performed in cells maintained for 3 days in culture (preconfluent cells). All experimentation products were diluted in DMSO (final concentration of DMSO was lesser than 0.1 %).

*Lactobacillus Plantarum (*ATCC® number 8014™) were provided by the Department of Microbiology of the University of Barcelona and growth in deMan, Rogosa and Sharpe (MRS) agar at 37 ºC and 5 % CO_2. After 24 h in culture, bacteria were scrapped for lysis and protein determination.

Cell Growth and [^{3}H]-thymidine Incorporation Assays

The effect of the treatments was assessed on Caco-2 cells clusters in 24-well plates (1.5-2 x 10^4 cells/cm^2). Cells were cultured for 96 h in DMEM medium supplemented with 10 % FBS. Then, cells were incubated for 48 h in the presence of both compounds. Finally, cells were washed, trypsinized and counted under a microscope using ethidium bromide/acridine orange staining to count cells and to assess viability.[24]

DNA synthesis in Caco-2 was assessed by measuring the incorporation of [^{3}H]-thymidine. Cells were seeded in 24-well culture plates (1.5-2 x 10^4 cell/cm^2) and cultured for 96 h in DMEM medium supplemented with 10 % FBS. Then, cells were incubated for 48 h in the presence of T-Rv or T-Pc at different concentration and 0.1 µCi/mL of [^{3}H]-thymidine. After 48 h [^{3}H]-thymidine-containing media were aspirated and cells were washed twice with 0.5 % BSA in PBS for [^{3}H]-thymidine elimination. Washed cells were lysed with 300 µL of 1 % Triton X-100 in PBS and added to a vial containing 3 mL of scintillation cocktail. Radioactivity present in cell fraction was measured with a liquid scintillation counter (Packard Tri-Carb 1500, GMI Inc.).

Flow Cytometry Cell Cycle Analysis

Caco-2 cells were seeded in 60 mm dishes (1.5 x 10^4 cells/cm^2), and 96 h after culture cells were then incubated by 48 h in 10 % FBS DMEM containing the treatments. Thereafter, cells were trypsinized, fixed with 70 % ethanol, and stored at 4 °C for at least 2 h. Next, low molecular weight DNA was extracted from cells, which were stained for 1 h at room temperature with a 20 µg/mL propidium iodide solution in PBS containing 0.1 % Triton X-100 and 0.2 mg/mL DNase-free RNase A. Cells were analyzed on an Epics XL flow cytometer (Coulter Corporation). DNA was analyzed (ploidy analysis) on single fluorescence histograms using Multicycle software (Phoenix Flow Systems).

Tunel Assay

Degradation of chromosomal DNA was evaluated with TUNNEL method using a MebStain Apoptosis Kit (MBL Int.). After 96 h in culture, Caco-2 cells were cultivated in media containing 10 % FBS DMEM with treatments for 48 h. Next, cells were fixed with 4 % paraformaldehyde and permeabilized with 70 % ethanol. Thereafter, 3′-OH DNA ends generated by DNA fragmentation were labeled with fluorescein-dUTP, mediated by terminal deoxynucleotidyl transferase, and were analyzed on an Epics XL flow cytometer (Coulter Corporation).

Total Antioxidant Activity and Reducing Power

We selected different concentration of any product and applied an Antioxidant Assay Kit (Cayman Chemical). This method is based on the ability of antioxidants in the sample (products diluted in PBS) to inhibit the oxidation of 2,2'-azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) to $ABTS^{\cdot+}$ by metmyoglobin.[25] The capacity of the antioxidants in the sample to prevent ABTS oxidation is compared with that of Trolox, a water-soluble tocopherol analogue, and is quantified as molar Trolox equivalents.

Total reducing power was determined according to the method of Oyaizu.[26] The first step was mix 40 µL of the sample solution with 200 µL of phosphate buffer (0.2 M, pH 6.6) and 200 µL of 1% potassium ferricyanide. The mixture was then incubated at 50 °C for 20 minutes. Afterwards, 200 µL of 10 % trichloroacetic acid was added to mixture. Finally, 60 µL of 0.167 % ferric chloride was added and incubated at room temperature for 50 minutes. Increased absorbance at 690 nm of the reaction mixture indicated increase in reducing power. Results were compared with a quercetin standard curve.

α-Glucosidase and β-Glucosidase Activity Assays

For enzymatic determination was necessary to extract proteins from cell and bacteria cultures. Caco-2 cells were scrapped with EDTA, centrifuged and resuspended in PBS, and bacteria were scrapped only with a handle and resuspended in PBS. After, cells and bacteria were lysed mechanically and sonicated (Ultrasonic processor UP200S, Hielscher Ultrasonic, Germany) to obtain a protein suspension without affect the enzymatic activity of the sample. The protein concentration was determined in the homogenate by the Bradford method[27] using the Bio-Rad protein assay kit with BSA as standard.

Enzymatic activity was measured by a spectrophotometric assay that measure the release of 4-nitrophenol from 4-nitrophenyl-α-D-glucopyranoside or 4-nitrophenyl-β-D-glucopyranoside (*p*-NPG, 5 mM), respectively, in citrate buffer (5 mM sodic citrate, 5 mM citric acid, pH 4.8). Samples (cellular and bacterial lysated), α-glucosidase and β-glucosidase standard with *p*-NPG were incubated 1 to 2 h at 37 ºC and the reaction was stopped with 1 M sodium carbonate. All tubes were analyzed at 405 nm with a Shimadzu UV-160A spectrophotometer.

Liquid Chromatography-Mass Spectrometry Analysis

Culture medium samples were filtered using 0,45 µm polytetrafluoroethylene filters, and then 15 µL of culture medium were injected. The analysis was performed based on the method previously described by Urpi-Sarda et al.[28] with some modifications. Briefly, a Phenomenex Luna C_{18} 50 mm x 2.0 mm 5 µm column was used for the HPLC analysis coupled to a single quadrupole mass spectrometer equipped with a turbo ion spray source working in negative mode. The compounds were separated using 0,5 mL/L acetic acid as mobile phase A and 700 mL/L acetone, 300 mL/L acetonitrile with 0,4 mL/L acetic acid as mobile phase B. A non-linear gradient was perform: 0 min, 90 % A; 0-1 min, 70 % A; 1-5 min, 0 % A; 5-5,6 min, 0 % A; 5,6-8 min, 90 % A; 11 min, 90 % A. The flow rate was 0.5 mL/min and the column was maintained at 40 ºC to improve the chromatographic separation. The MS parameters were: Capillary voltage -3500 V, curtain gas (N_2) 12 (arbitrary units), nebulizer gas (N_2) 10 (arbitrary units), focusing potential -200 V, entrance potential -10 V, declustering potential -50 V, dry gas (N_2) heated at 400 ºC and introduced at flow rate of 6000 cm^3/min. SIM (Single Ion Monitoring) mode was used to quantify *cis*-Rv (C-Rv) and T-Rv and *cis*-Pc (C-Pc) and T-Pc. The dwell time applied for piceid (m/z 389) was 500 msec and for taxifolin and resveratrol (m/z 227,303,185,143) was 250 msec. Quantification of T-Rv and T-Pc were carried out using a six-point weighted calibration curve of the standards with taxifolin as internal standard. C-Rv and C-Pc were quantified using an isomerized calibration curve, since T-Rv and T-Pc that were isomerized by UV exposure.

Data Analysis

Results are expressed as mean ± standard error of the mean. Differences between non-treated and treated cells were tested by using Student's t-test. Differences of $P < 0.05$ were considered significant.

Results

Our results shown that T-Rv has higher antioxidant and reducing power than Trolox and quercetin, respectively. Interestingly, we observed that T-Pc also show antioxidant activity similar to that of T-Rv, although without reducing power. Moreover, we observed an additional effect of both together (Table 1). Biological free radicals are among the underlying pathophysiological causes of the development of cancer.[29,30] Due to T-Pc presents a direct antioxidant activity our next aim was to examine its antiproliferative action on human colon cancer cell line.

To determine the effect of T-Pc and T-Rv on cell growth induced by FBS 10 %, non-differentiated Caco-2 cells were incubated with these polyphenols. As shown in Figure 1A, T-Pc and T-Rv significantly inhibited Caco-2 cell growth in a concentration-dependent manner. Considering that Henry et al.[31] reported that intestinal epithelial cells can hydrolyze T-Pc and release T-Rv. It is important to study whether the antiproliferative effect induced by T-Pc is consequence of T-Rv release from T-Pc. When cells were treated with T-Pc combined with an β-glucosidase inhibitor, gluconolactone,[32] it was observed that the presence of the inhibitor did not reverse the effect induced by T-Pc in a significantly manner at 2 mM (Figure 1B). The effect of T-Pc and T-Rv on non-differentiated Caco-2 cell DNA synthesis was also examined by [^{3}H]-thymidine incorporation in cell cultures. At 10 μM T-Rv inhibited DNA synthesis more than T-Pc at the same concentration, but at 25 μM both induce around 50 % of inhibition of [^{3}H]-thymidine uptake by Caco-2 cells after 48 h exposure (Figure 2A). When the experiment was carried out in presence of gluconolactone at 2 mM, we do not observe a significantly reversion of the effects induced by T-Pc on cellular [^{3}H]thymidine incorporation (Figure 2B). Moreover, we observed that T-Rv and T-Pc added their effects on Caco-2 cell growth inhibition (Figure 1A and Figure 2A).

The lack of effect of β-glucosidase inhibitor on the inhibition of Caco-2 cell growth and DNA synthesis induced by T-Pc suggests that T-Pc could have a direct action on

these events. This hypothesis is supported by the low β-glucosidase activity presents in non-differentiated and differentiated Caco-2 cells regarded to *Lactobacillus Plantarum* β-glucosidase activity (Table 2).[33] To corroborate the lack of T-Pc deglycosilation in our experimental conditions, Caco-2 cell cultures were incubated with T-Pc and we observed a constant concentration of the polyphenol over time (0-24 h), and a slight concentration of C-Pc, probably because the commercial product have a 95 % of purity (Figure 3A). When cell cultures were treated with T-Rv, we found 95 % of T-Rv at 0 time and this concentration decreased to 9 % the next 6 h, probably as consequence of its metabolization by intestinal epithelial cells (Figure 3B).

To analyze the effect of T-Pc and T-Rv on cell cycle, the distribution of cells in each phase of the cell cycle was quantified by determining DNA content via flow cytometry, after 48 h exposure in the presence of FBS 10 % and polyphenols. Cell cycle analysis of T-Rv treated cells showed that this polyphenol induced a significant accumulation of cells in the S phase with a consequent reduction in the percentage of cells in the G_0/G_1 phase at 25 μM. Interestingly, under the same conditions, T-Pc led to a significant accumulation of cells in the G_0/G_1 phase and reduced the percentage of cells in the S phase at concentrations between 10 and 25 μM (Figure 4A).

We did not observe hypodiploid DNA peak, characteristic of apoptosis, in these experimental conditions (Figure 4B). However, we measured DNA fragmentation induced by T-Pc and T-Rv as an index of their apoptotic activity. Figure 5 shows that the T-Pc induced marked DNA fragmentation (50 %) at 50 μM, whereas T-Rv had a lower effect at different concentrations (25-100 μM).

Discussion

T-Pc has diverse biological effects[15,16] that could be attributed to T-Rv released as consequence of deglycosidation of T-Pc by intestine and/or liver. In this way, Kineman et al.[34] reported that T-Rv incorporated into diet reduced the number of aberrant crypt foci formation in experimental colon cancer whereas diet prepared with transgenic alfalfa rich in T-Pc was not effective. However, diets containing T-Pc with exogenous β-glucosidase did significantly reduce aberrant crypt foci, suggesting that T-Rv released from T-Pc was effective in this murine model of carcinogenesis.[34] Our results show that T-Pc has a direct antioxidant activity *in vitro* in a similar extent than T-Rv as was recently reported by Medina et al.[35] The hydroxyl group at the 4'-position is relevant for the antioxidant efficiency of T-Rv and it is maintained in T-Pc and consequently keeps the antiradical activity. Moreover, our results demonstrated that T-Pc is antiproliferative on non-differentiated Caco-2 cells similar to T-Rv in agreement with Ha et al.[36] that reported the inhibition of L1210 (mouse lymphocytic leukemia cell line) and K562 (human erythromyeloblastoid leukemia cell line) cells by T-Pc.

Caco-2 cell line spontaneously differentiates into polarized enterocytes expressing brush border enzymes typical of small intestine epithelial cells such as disaccharidases. In our experimental conditions, we observed a very low β-glucosidase activity in these cells, whereas these non-differentiated intestinal epithelial cells present a high α-glucosidase activity. Interestingly, we did not observe an appreciable deglycosidation of T-Pc by non-differentiated Caco-2 cell cultures. All together suggest that T-Pc is not appreciably deglycosilated in our experimental conditions and might have direct antiproliferative action in Caco-2 cell cultures without T-Rv implication in these events. Moreover, our findings show that T-Pc effect was added to T-Rv action on Caco-2 cell growth inhibition.

The flow cytometry results indicated a significant reduction in the number of cells in the G_0/G_1 phase of the cell cycle by T-Rv, whereas the S phase population increased in

agreement with previous reports.[37] However, these effects were specific for T-Rv because T-Pc led to the accumulation of Caco-2 cells in the G_0/G_1 phase. These findings suggest different mechanism of action of T-Rv and T-Pc on the cell cycle, with a similar final effect on Caco-2 cell growth. Interestingly, T-Pc action on Caco-2 cell cycle was similar to the effects induced by T-Rv metabolites (sulphates and glucuronides).[37] Thus, T-Rv and T-Pc may act in different and redundant molecular targets and pathways involved in the regulation of the cell cycle. Additional studies are necessary to identify these targets to T-Pc.

Previous studies have shown that T-Rv induces apoptosis at concentration of 100 μM in colorectal cancer cells.[11] It should be noted that T-Pc at 50 μM decreased cell number respect to control conditions without FBS in a higher extent than T-Rv in our experimental conditions. These findings suggest a possible cytotoxic or pro-apoptotic effect of T-Pc. This hypothesis was confirmed due to it was observed that T-Pc at 50 μM induced DNA fragmentation, whereas T-Rv had a minor effect at this concentration. T-Pc is diffused to most tissues immediately after oral administration with a peak level at 10 min. The maximum level of T-Pc was detected in stomach (169 μg/g) and intestine (109 μg/g) after an oral dose of 50 mg/Kg to Wistar rats.[38] Recently, Rotches-Ribalta reported that total human plasma resveratrol/piceid (free and metabolites) after moderate red wine consumption reached around μM concentrations.[39] Considering these pharmacokinetic data, our findings suggested that T-Pc concentrations reached in colon mucosa after oral T-Pc intake might have an appreciably anti-proliferative/pro-apoptotic actions. However, additional investigation is needed into biological activity of piceid and piceid plus resveratrol on intestinal epithelial proliferation in *in vivo* models.

Conclusion

To our knowledge this is the first time that an antiproliferative effect of T-Pc, through the control of cell cycle and apoptosis, has been described in intestinal epithelial cells. Results in agreement with recent studies that reported an anti-proliferative effect of T-Pc on human liver and breast cancer cells[40] and lung cancer cells.[41] Moreover, these findings suggest that T-Pc must not be considered exclusively as T-Rv reservoir and can raise biological effect by itself. Furthermore, a synergistic action of T-Rv and T-Pc can be considered. These data prompted us to investigate in further detail the mechanism of action of T-Pc on the cell cycle machinery in the future.

Conflict of interest

Authors have no conflict of interest affecting the conduct or reporting of the work submitted.

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References

(1) A.I. Romero-Perez, R.M. Lamuela-Raventos, C. Andres-Lacueva and M.C. de La Torre-Boronat, J. Agric. Food Chem., **2001**, *49*, 210-215.

(2) G.J. Soleas, E.P. Diamandis and D.M. Goldberg, Adv. Exp. Med. Biol., **2001**, *492*, 159-182.

(3) M. Sovak, J. Med., Food **2001**, *4*, 93-105.

(4) R. Zamora-Ros, C. Andres-Lacueva, R.M. Lamuela-Raventos, T. Berenguer, P. Jakszyn, C. Martinez, M.J. Sanchez, C. Navarro, M.D. Chirlaque, M.J. Tormo, J.R. Quiros, P. Amiano, M. Dorronsoro, N. Larranaga, A. Barricarte and E. Ardanaz, C.A. Gonzalez, Br. J. Nutr., **2008**, *100*, 188-196.

(5) M.T. Ribeiro de Lima, P. Waffo-Teguo, P.L. Teissedre, A. Pujolas, J. Vercauteren, J.C. Cabanis and J.M. Merillon, J. Agric. Food Chem., **1999**, *47*, 2666-2670.

(6) F. Mattivi, F. Reniero and S. Korhammer, J. Agric. Food Chem., **1995**, *43*, 1820-1823.

(7) X. Vitrac, J. Monti, J. Vercauteren, G. Deffieux and J.M. Mérillon, Anal. Chim. Acta, **2002**, *458*, 103-110.

(8) Y.C. Chukwumah, L.T. Walker, M. Verghese, M. Bokanga, S. Ogutu and K. Alphonse, J. Agric. Food Chem., **2007**, *55*, 285-290.

(9) C. Gentile, L. Tesoriere, D. Butera, M. Fazzari, M. Monastero, M. Allegra and M.A. Livrea, J. Agric. Food Chem., **2007**, *55*, 643-648.

(10) A.M. Rimando, W. Kalt, J.B. Magee, J. Dewey and J.R. Ballington, J. Agric. Food Chem., **2004**, *52*, 4713-4719.

(11) N.F. Trincheri, G. Nicotra, C. Follo, R. Castino and C. Isidoro, Carcinogenesis, **2007**, *28*, 922-931.

(12) K.P.L. Bhat, J.W. Kosmeder, J.M. Pezzuto, Antioxid. Redox Signal **2001**, *3*, 1041-1064.

(13) J.M. Merillon, B. Fauconneau, P.W. Teguo, L. Barrier, J. Vercauteren and F. Huguet, Clin. Chem., **1997**, *43*, 1092-1093.

(14) M. Varache-Lembège, P. Waffo-Téguo, T. Richard, J.P. Monti, G. Deffieux, J. Vercauteren, J.M. Mérillon and A. Nuhrich, A. Med. Chem. Res., **2000**, *10*, 253-267.

(15) Y. Cheng, H.T. Zhang, L. Sun, S. Guo, S. Ouyang, Y. Zhang and J. Xu, Brain Res., **2006**, *1110*, 193-200.

(16) J. Du, L.N. Sun, W.W. Xing, B.K. Huang, M. Jia, J.Z. Wu, H. Zhang and L.P. Qin, Phytomedicine, **2009**, *16*, 652-658.

(17) J. Martinez and J.J. Moreno, Biochem. Pharmacol., **2000**, *59*, 865-870.

(18) J.J. Moreno, J. Pharmacol. Exp. Ther., **2000**, *294*, 333-338.

(19) K. Nemeth, G.W. Plumb, J.G. Berrin, N. Juge, R. Jacob, H.Y. Naim, G. Williamson, D.M. Swallow and P.A. Kroon, Eur. J. Nutr., **2003**, *42*, 29-42.

(20) A.J. Day, J.M. Gee, M.S. DuPont, I.T. Johnson and G. Williamson, Biochem. Pharmacol., **2003**, *65*, 1199-1206.

(21) A.P. Wilkinson, J.M. Gee, M.S. Dupont, P.W. Needs, F.A. Mellon, G. Williamson and I.T. Johnson, Xenobiotica, **2003**, *33*, 255-264.

(22) C. Henry-Vitrac, A. Desmouliere, D. Girard, J.M. Merillon and S. Krisa, Eur. J. Nutr., **2006**, *45*, 376-382.

(23) A.J. Day, M.S. DuPont, S. Ridley, M. Rhodes, M.J. Rhodes, M.R. Morgan and G. Williamson, FEBS Lett., **1998**, *436*, 71-75.

(24) D.R. Parks, V.M. Bryan, V.T. Oi and L.A. Herzenberg, Proc. Natl. Acad. Sci. U. S. A., **1979**, *76*, 1962-1966.

(25) N.J. Miller and C.A. Rice-Evans, Free Radic. Res., **1997**, 26, 195-199.

(26) M. Oyaizu, Jpn. J. Nutr., **1986**, *44*, 307-314.

(27) M.M. Bradford, Anal. Biochem., **1976**, *72*, 248-254.

(28) M. Urpi-Sarda, R. Zamora-Ros, R. Lamuela-Raventos, A. Cherubini, O. Jauregui, R. de la Torre, M.I. Covas, R. Estruch, W. Jaeger and C. Andres-Lacueva, Clin. Chem., **2007**, *53*, 292-299.

(29) S.U. Mertens-Talcott, J.H. Lee, S.S. Percival and S.T. Talcott, J. Agric. Food Chem., **2006**, *54*, 5336-5343.

(30) T.E. Kraft, D. Parisotto, C. Schempp and T. Efferth, Crit. Rev. Food Sci. Nutr., **2009**, *49*, 782-799.

(31) C. Henry, X. Vitrac, A. Decendit, R. Ennamany, S. Krisa and J.M. Merillon, J. Agric. Food Chem., **2005**, *53*, 798-803.

(32) M.L. Shulman, S.D. Shiyan and A. Khorlin, Biochim. Biophys. Acta, **1976**, *445*, 169-181.

(33) A.B.F. Sestelo, M. Poza and T.G. Villa, World Journal of Microbiology and Biotechnology, **2004**, *20*, 633-637.

(34) B.D. Kineman, A. Au, N.L. Paiva, M.S. Kaiser, E.C. Brummer and D.F. Birt, Nutr. Cancer, **2007**, *58*, 66-74.

(35) I. Medina, D. Alcantara, M.J. Gonzalez, P. Torres, R. Lucas, J. Roque, F.J. Plou and J.C. Morales, J. Agric. Food Chem., **2010**, *58*, 9778-9786.

(36) T. Ha do, Q.C. Chen, T.M. Hung, U.J. Youn, T.M. Ngoc, P.T. Thuong, H.J. Kim, Y.H. Seong, B.S. Min and K. Bae, Arch. Pharm. Res., **2009**, *32*, 177-183.

(37) C.E. Storniolo and J.J. Moreno, Food Chem., **2012**, *134*, 1385-1391.

(38) C. Lv, L. Zhang, Q. Wang, W. Liu, C. Wang, X. Jing and Y. Liu, Biomed. Chromatogr., **2006**, *20*, 1260-1266.

(39) M. Rotches-Ribalta, C. Andres-Lacueva, R. Estruch, E. Escribano, M. Urpi-Sarda, Pharmacol. Res., **2012**, *66*, 375-382.

(40) Y. Zhang, Z. Zhuang, Q. Meng, Y. Jiao, J. Xu and S. Fan, Oncol Lett., **2014**, *7*, 295-301.

(41) D. Su, Y. Cheng, M. Liu, D. Liu, H. Cui, B. Zhang, S. Zhou, T. Yang and Q. Mei, PLoS One., **2013**, *8*, e54505.

Fig. 1. Effect of T-Rv and T-Pc on Caco-2 cell growth induced by FBS 10 %. (A) Cells were treated with individual (1-100 μM) and mixed (10 + 10 μM) T-Rv and T-Pc for 48 h and counted. Values are mean ± SEM (n = 12-20). (B) Effect T-Pc (25 μM) with and without the inhibitor gluconolactone (Gluc) at 2 mM on Caco-2 cell growth induced by FBS 10 %. Cells were treated for 48 h with treatments and counted. Negative control of proliferation was performed in absence of FBS (-FBS). Values are mean ± SEM (n = 14-15). * $P < 0.05$ versus control group (cells cultured in presence of FBS). # $P < 0.05$ versus cells cultured with T-Rv.

Fig. 2. Effect of T-Rv and T-Pc on Caco-2 DNA synthesis induced by FBS 10 %. (A) Cells were treated with individual (10-25 μM) and mixed (10 + 10 μM) T-Rv and T-Pc for 48 h in presence of [^{3}H]-thymidine and after [^{3}H]-thymidine uptake assayed. Values are mean ± SEM (n = 7-14). (B) Effect T-Pc (25 μM) with and without the inhibitor gluconolactone (Gluc) at 2 mM on Caco-2 DNA synthesis induced by FBS. Cells were treated for 48 h with treatments in presence of [^{3}H]-thymidine and [^{3}H]-thymidine uptake assayed. Values are mean ± SEM (n = 8-10). Negative control of proliferation was performed in absence of FBS (-FBS). * $P < 0.05$ versus control group (cells cultured in presence of FBS).

Fig. 3. T-Rv and T-Pc levels in Caco-2 supernatant cultures. Non-differentiated Caco-2 cells were incubated with T-Pc (A) or T-Rv (B) at 25 μM. 0, 6, 15 and 24 h after we take samples of culture supernatant and T-PC, T-Rv and their *cis*- isomers were quantified by HPLC. Results are expressed as percentage of variation respect the total content of T-Pc or T-Rv and their *cis*-isomers at baseline. Values are mean ± SEM of two experiments performed in duplicate.

Fig. 4. Effect of T-Rv and T-Pc on Caco-2 cell cycle. Non-differentiated cell cultures were incubated with T-Rv or T-Pc (10-25 μM) for 48 h and the flow cytometric analysis of the cell cycle was conducted, we show a cell cycle distribution of a representative experiment (A). Grey, white and black bars represent the percentage of cells in S, G_2/M and G_0/G_1 phase, respectively (B). Negative control of proliferation was performed in absence of FBS (-FBS).

Fig. 5. Effect of T-Rv and T-Pc on apoptosis. Non-differentiated Caco-2 cells were incubated with polyphenols (25-100 μM) for 48 h and DNA fragmentation was measured. Values are mean ± SEM (n = 7-14). * $P < 0.05$ versus negative control group (cells cultured in presence of FBS 10%). As positive controls we used staurosporine (1 μM).

Table 1. Total antioxidant activity and reducing power of T-Rv and T-Pc.

	Total antioxidant activity (mM)	Reducing power (mg/mL)
T-Rv (50 μM)	0.183 ± 0.01	7.385 ± 0.07
T-Rv (100 μM)	0.225 ± 0.03	ND
T-Pc (50 μM)	0.205 ± 0.03	0.02 ± 0.01
T-Pc (100 μM)	0.230 ± 0.01	ND
T-Rv (100 μM)+ T-Pc (100 μM)	0.462 ± 0.1	ND

Data are the mean ± SEM of three experiments performed in duplicate. Total antioxidant activity is expressed as equivalents of Trolox (mM). Reducing power is expressed as equivalents of quercetin (mg/mL). ND, not determined.

Table 2. Glucosidase activities of non-differentiated (preconfluent) and differentiated Caco-2 cells cultured in presence of FBS 10 %.

	(μUnits of enzyme / μg of protein / minute)	
	α-Glucosidase	β-Glucosidase
Non-differentiated cells	2.13 ± 0.3	0.11 ± 0.02
Differentiated cells	6.52 ± 0.4*	0.11 ± 0.01
Lactobacillus Plantarum	ND	1.17 ± 0.09

Data are the mean ± SEM of three experiments performed in duplicate. * $P < 0.05$ vs non-differentiated cells. ND, not determined.

FIGURE 1

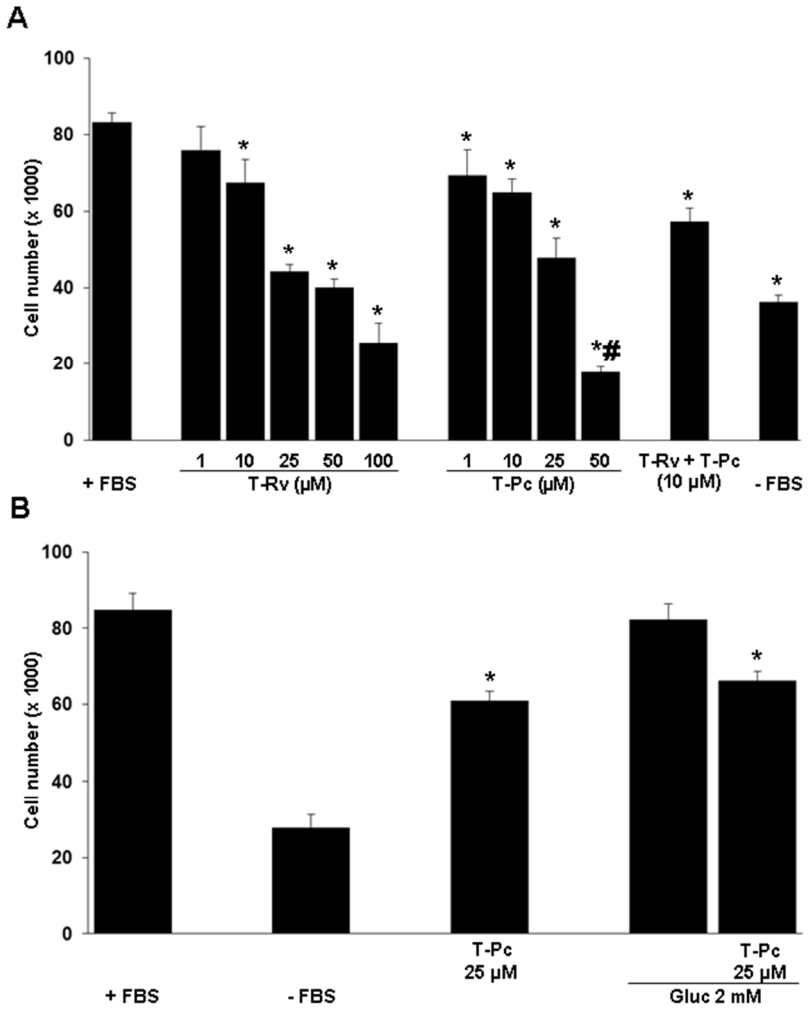

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FIGURE 2

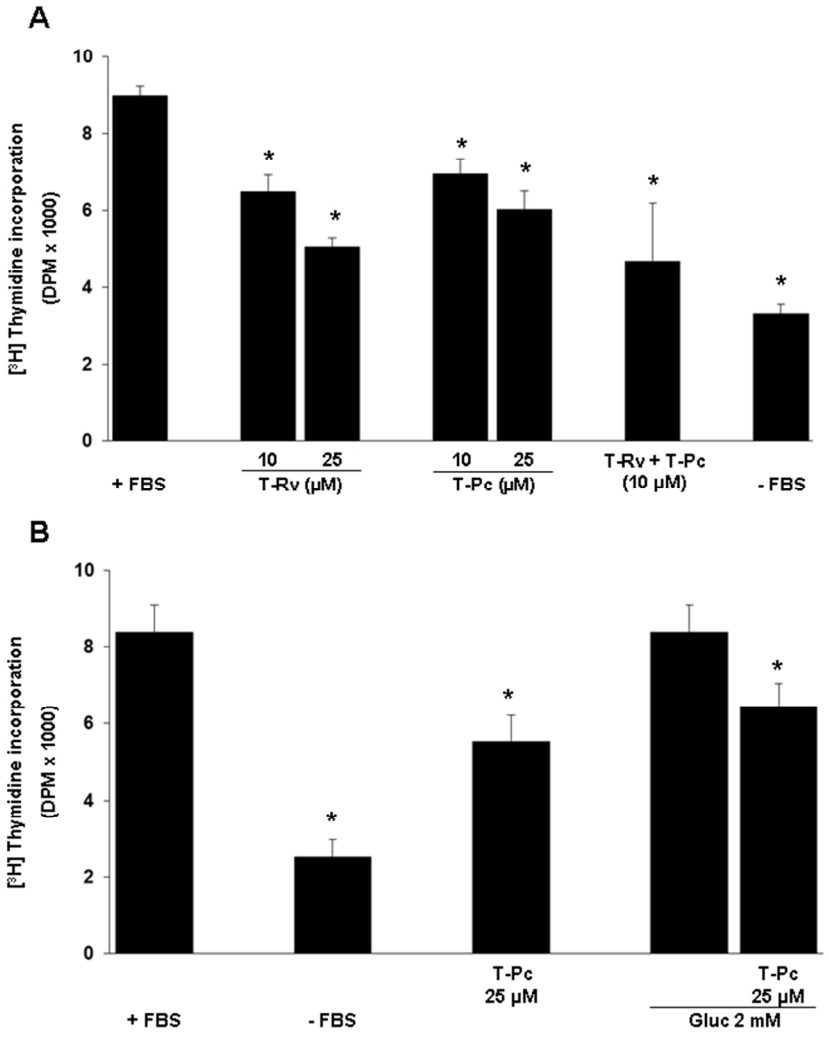

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FIGURE 3

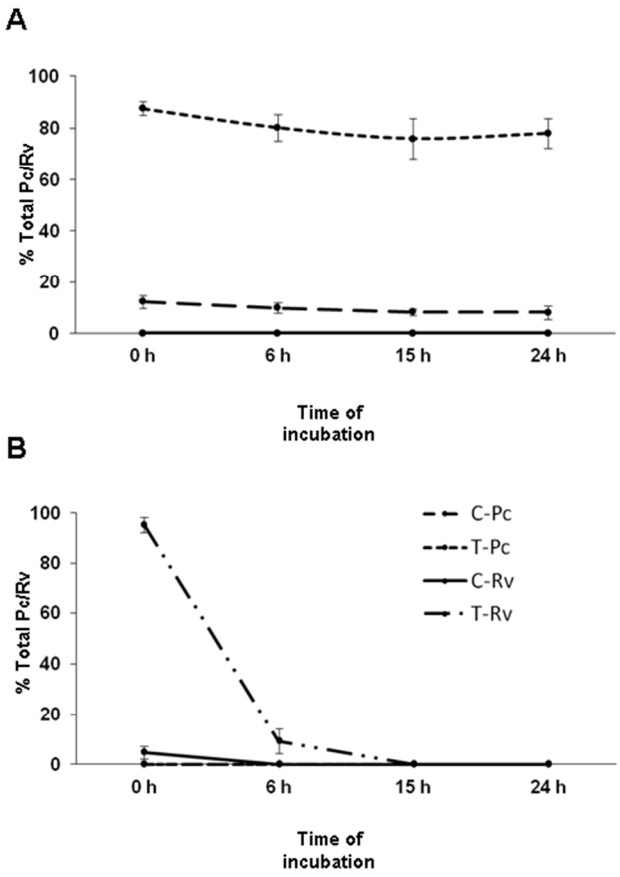

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FIGURE 4

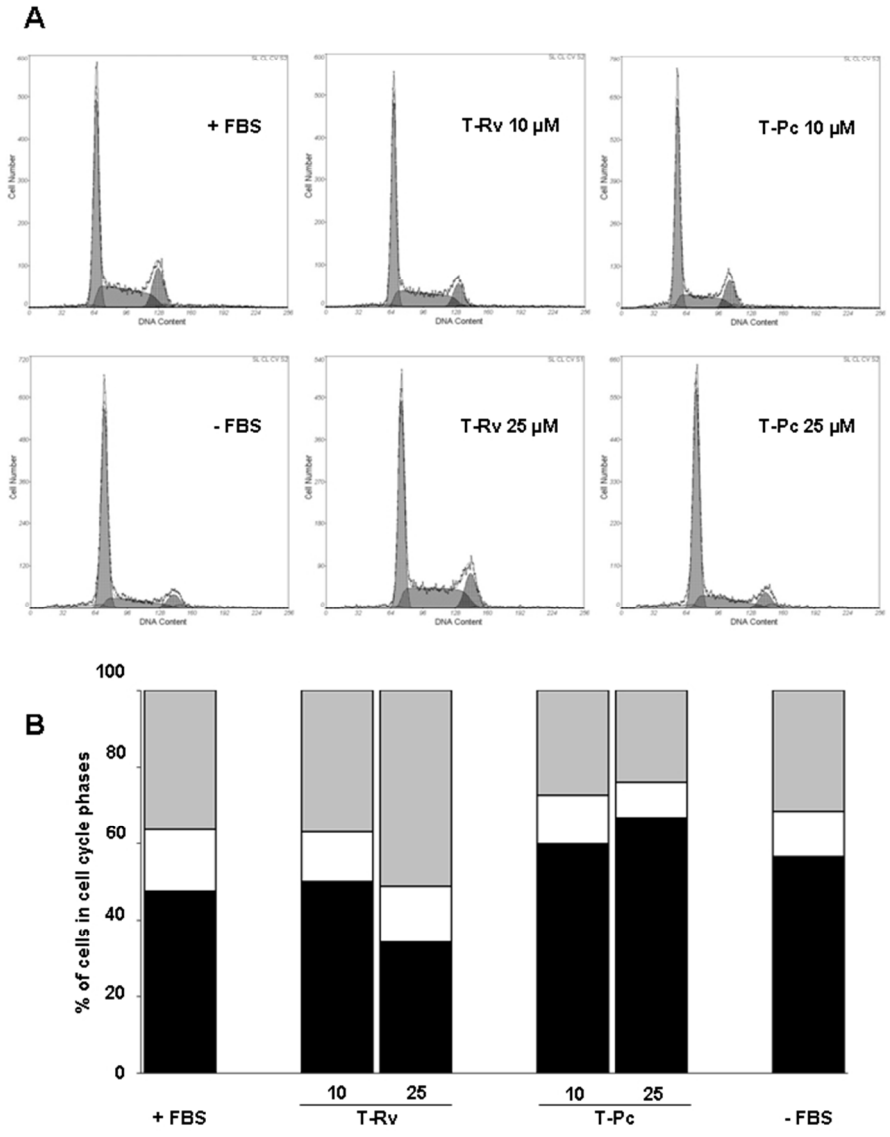

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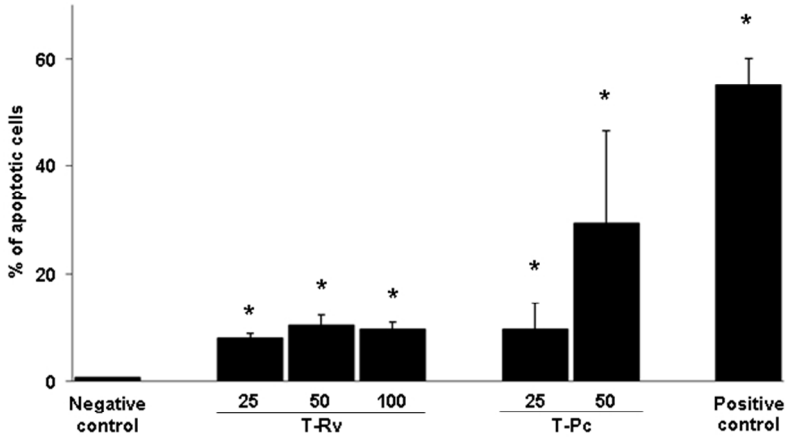
FIGURE 5



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