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Title: Nutraceutical composition of three pine bark extracts and their antiproliferative effect on Caco-2 cells

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Keywords: Pine bark extracts; Antiproliferative effect; Antioxidant activity; Caco-2 cells; Functional foods; ROS

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Abstract: The use of vegetal materials that go unused or by-products of the food industry, for the development of functional foods is very interesting. Pine bark extracts have been useful in medicine and in functional foods, yet, little is known about its antiproliferative properties. In this work, the activity of bark extracts from different Pinus species on human colorectal adenocarcinoma (Caco-2) cells was studied. All these extracts induce cell-cycle arrest and apoptosis in Caco-2 cells through alteration of the mitochondrial membrane potential, release of cytochrome c to cytoplasm and caspase 3 activation. In addition, a significant decrease in ROS generation was also observed in the presence of the extracts tested. The results obtained in this work show that these extracts could be interesting in order to elaborate functional foods and not only for improving their antioxidant properties but also for playing an important role in the treatment of colorectal carcinoma.

June 26, 2018

Professor Udenigwe,
Associate Editor
Journal of Functional Foods

Dear Dr. Udenigwe,

We are pleased to know that our manuscript "Nutraceutical composition of three pine bark extracts and their antiproliferative effect on Caco-2 cells" (JFF-D-17-02639) has been revised for publication in Journal of Functional Foods. We have made the changes to the text recommended by the reviewers. We are also grateful for your suggestions.

I hope that everything is in order. If you have any questions, feel free to contact me.
Thanking you in advance,

Yours faithfully,

Carmen Ancín-Azpilicueta

MANUSCRIPT JFF-D-17-02639

RESPONSE TO REVIEWER 1 AND CHANGES WE HAVE MADE TO THE MANUSCRIPT

Firstly, we want to thank you for your comments as well as for your revision of the manuscript. Below we have made a detailed reply to each of your observations. We have also corrected the manuscript in accordance with your suggestions.

Point by point response to reviewer:

Referee´s comments have been written in italics to distinguish them from our own texts.

Reviewer #1:

The aim of this work is to evaluate the anti-proliferative effects and antioxidant activity of different pine bark extracts. The research that is presented within this paper is original and has practical applications. The work is well design and presented. The introduction chapter provides good background information for the tasks of this paper and motivation for this work is well explained. Results are well discussed. However, to further improve the quality of the manuscript some modifications are proposed:

-This manuscript requires careful editing for English language including grammar and choice of words.

In line with your suggestion the English language, including grammar, throughout the manuscript has been reviewed.

-Page 4, Lines 60-88: Please reduce the information related to Pinus pinaster. Some information is not necessary in this section.

In line with your suggestion, information shown in lines 60-88 of the original manuscript has been reduced. All the eliminated lines are now in red and crossed out.

-Can you provide more information related to the standards (purity, origin, where they were purchased)?

The purity and origin of the standards are detailed below:

Taxifolin: purity ≥ 90%, Sigma-Aldrich (Madrid, Spain)
Catechin: purity≥ 99%, Sigma-Aldrich
Procyanidin B1, B2: purity ≥ 90% Extrasynthese (Lyon, France)
Procyanidin A2: purity ≥ 99% Extrasynthese

This information related to the standards used, has now been inserted in the revised manuscript (page 7, lines 138-140).

-Total antioxidant activity: The authors used only one assay (ABTS) to determine the antioxidant capacity. Recently, it was recommended several times to use at least two different test systems for investigations of antioxidant capacity. The results of this assay strongly depend on the reaction principle, the radical used and others.

The aim of this work was to compare the antioxidant capacity of different bark extract samples under the same conditions. The use of several methods would have been important if these extracts had been added to a food and if we wanted to know the antioxidant capacity in this enriched food. However, just to measure the antioxidant capacity of an extract, the result obtained from a single method such as ABTS is sufficient. We chose the ABTS method because has been accepted as a reliable method both in food analysis and clinical research, given that it provides results over a wide range of pH and it serves for both hydrophilic and lipophilic molecules. The results which are obtained of this method are reproducible and show several maximums of absorption. On the other hand, the individual antioxidants present in the extract were determined by HPLC. This provides very important information as it is observed that the antioxidant capacity coincides with the total concentration of these substances. However, we appreciate your suggestion and we will take it into account in future research work.

-Please provide more information about the Sánchez-de-Diego protocol.

This information has been improved and expanded in pages 9-11, lines 195-227 of the revised manuscript. A new reference has been added.

> *Christensen ME, Jansen ES, Sanchez W, Waterhouse NJ. Flow cytometry based assays for the measurement of apoptosis-associated mitochondrial membrane depolarization and cytochrome c release. Methods. 2013 Jun 1; 61(2):138-45.*

-Page 11, Lines 241-248: provide this information in Material and Methods.

This information has been provided in the Material and Methods section (pages 7-8, lines 152-159 of the revised manuscript).

-Page 15: "Effect of procyanidins on cellular viability". Some information from this section should be provided in Material and Methods. You do not explain anything related to this section in Material and Methods.

Information related to the method used to determine the effect of procyanidins on cell viability has been added in Material and Methods (page 8, lines 172-173 of the revised manuscript).

MANUSCRIPT JFF-D-17-02639

RESPONSE TO REVIEWER 2 AND CHANGES WE HAVE MADE TO THE MANUSCRIPT

First of all, we want to thank you for your comments as well as for your revision of the manuscript. Your suggestions have helped to improve the quality of the paper. Below we have made a detailed reply to each of your observations. We have also corrected the manuscript in accordance with your suggestions.

Point by point response to reviewer:

Referee´s comments have been written in italics to distinguish them from our own texts.

Reviewer #2: The work by Gascon et al. described the nutraceutical composition of extracts from the bark of three different Pinus species (Pinus pinaster, Pinus pinea, Pinus halepensis), and their antiproliferative and antioxidative effect on human colorectal adernocarcinoma (Caco-2) cells. The work is original and has the potential to be important to nutraceutical research and readers. However, the quality of English needs to be improved to properly convey the findings and ideas. Also, explanation/discussion of the results (Tables and Figures) needs to be improved. I would recommend this article for Journal of Functional Foods upon addressing the specific points below.

1. Authors should include a graphical abstract to convey the main message to readers.

In line with your suggestion a graphical abstract has been included in the revised manuscript.

2. Line 88: delete 'must' in the sentence

"Must" has been removed from the sentence on page 5 line 88 of the original manuscript.

3. Line 90: the claim is a bit vague. Authors should enhance it by clearly stating the other specific species which they claim to have been less studied.

We acknowledge the reviewer's recommendation. We agree that this paragraph was a bit confusing and we have changed it in order to improve its comprehension (page 5, lines 98-105 of the revised manuscript). We hope that with these changes that paragraph is now clearer.

4. Line 89-99: Authors need to revise the paragraph. The complexity of the paragraph is compounded specifically by line 95: Authors stated that "… the aim of this work was to determine whether 'these' pine extracts may inhibit the growth of human colon cancer cell lines (Caco-2) by apoptotic way". Which pine extract are the authors referring to? That of Yescin et al. since they make mention of "these"? Authors then moved on to state a different set of pines from what they discussed earlier, which happens to be the actual samples.

This paragraph has been modified just as we have explained in the previous section.

5. Line 203: statement should be revised as: "The amount of catechin in P. pinea and in P. pinaster was very similar…"

In line with your suggestion, we have reviewed and changed the sentence in the revised manuscript (page 12, lines 250-252 of the revised manuscript).

6. Line 209 - 211: This statement appears to be redundant as it has already been highlighted neatly in the first sentence in section 3.1, line 199. The main missing piece is the brief chemistry behind how Catechin acts as a powerful antioxidant. All other points stated can be clearly seen in the results (Table 1).

In accordance with the reviewer's request, we have eliminated the sentence that seemed like a repetition in regard to the sentence from line 199 of the original manuscript. We have added a new sentence and a new reference in which the mechanism of the antioxidant action is briefly described (page 12, lines 260-265 of the revised manuscript).

8. Line 233-238: Is there any possible reason for this observed phenomenon; why different trends in the amount of procyanidin B1 and B2 extracts in the pine samples?

The pine samples analyzed were from different species and, so, they could each have a different composition. There are not many studies that have analyzed the content of procyanidins in different species of pine bark but, for example, Jerez et al. (2009) found that procyanidins in *P. radiata* bark were more polymerized than *P. pinaster* bark procyanidins. Besides, Hellström et al. (2009) determined the content of procyanidins in a large number of vegetable food products, and found certain variability in the content of these flavan-3-ols in the same product. Disparity between the results can arise from natural variability such as genotype, differences in growing and harvesting conditions, climate, soil type, etc. (Duc et al., 1995; Cadot et al., 2006; Renard et al., 2007). This clarification and the reference to Hellström et al. (2009) have been inserted into the revised manuscript (page 13, lines 288-290 of the revised manuscript).

Cadot, Y.; Miñana Castelló, M. T.; Chevalier, M. Flavan-3-ol compositional changes in grape berries (*Vitis vinifera* L. cv Cabernet Franc) before veraison, using two complementary analytical approaches, HPLC reversed phase and histochemistry. Anal. Chim. Acta, 2006, 563, 65–75.

Duc, G.; Brun, N.; Merghem, R.; Jay, M. Genetic variation in tannin-related characters of faba-bean seeds (*Vicia faba* L.) and their relationship to seed-coat colour. Plant Breed. 1995, 114, 272–214.

Hellström JK, Törrönen AR, Mattila PH (2009) Proanthocyanidins in common food products of plant origin. J. Agric. Food Chem., 57, 7899–7906
Renard, C. M. G. C.; Dupont, N.; Guillermin, P. Concentrations and characteristics of procyanidins and other phenolics in apples during fruit growth. Phytochemistry 2007, 68, 1128–1138.

9. Line 237-238: Authors reported that "P. halepensis was the only one that had procyanidin A2 in its bark". Of what relevance is this finding, procyanidin A2 in only P. halepensis, to the aim of this study? Authors need to clearly highlight that.

This question is related to the previous one, i.e. different species of plants usually display a different phenol composition. In the research work of Hellström et al. (2009) which analyzes the content of procyanidins from different fruit and vegetables, *Prunus domestica* has A-type procyanidins while in *Prunus persica* only B-type procyanidins were found and the same thing occurs between different species of *Vaccinium* berries.

10. Line 238: Total antioxidant activity does not look similar in your results, Table 1, between P. pinea and P. pinaster contrary to the claim in line 238. There is indeed an observable difference in each pine sample antioxidant, with P. halepensis being the least. This corroborates with your data on the flavonoids extracts in Table 1.

We have changed the sentence in line 238 of the original manuscript with the aim of clarifying that *P. pinaster* has greater antioxidant capacity than *P. pinea* and the bark of *P. halepensis* displays the least antioxidant capacity (page 13, lines 292-293 of the revised manuscript).

11. Line 282-283: the statement is unclear. Based on your results, do you mean to say "It was observed that an increase in active caspase-3 was compatible with the induction of apoptosis in Caco-2 cells"

Following your suggestion, this paragraph has been changed (page 15, lines 336-338 of the revised manuscript).

12. Line 317: statement should be revised as: "…being slightly higher than…"

This paragraph has been improved (page 17, lines 371-373 of the revised manuscript).

13. Line 346-347: I suggest the sentence to be revise: "... important antioxidants such as taxifolin, catechin, procyanidin B1 (especially in P. pinea) and procyanidin B2 (especially in P. pinaster). Additionally, Procyanidin A2 was present only in P. halepensis."

The sentence in lines 346-347 from the original manuscript has been changed in line with your suggestion (page 18, lines 398-401 of the revised manuscript).

Highlights

- Pine extracts have anti-proliferative effect through mitochondrial apoptosis.
- A significant decrease in ROS generation was found in the presence of pine barks.
- *P. pinaster* was the one that showed the greatest biological activity.
- Pine barks are interesting for developing functional foods.

Nutraceutical composition of three pine bark extracts and their antiproliferative effect on Caco-2 cells

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ABSTRACT

The use of vegetal materials that go unused or by-products of the food industry, for the development of functional foods is very interesting. Pine bark extracts have been useful in medicine and in functional foods, yet, little is known about its antiproliferative properties. In this work, the activity of bark extracts from different *Pinus* species on human colorectal adenocarcinoma (Caco-2) cells was studied. All these extracts induce cell-cycle arrest and apoptosis in Caco-2 cells through alteration of the mitochondrial membrane potential, release of cytochrome c to cytoplasm and caspase 3 activation. In addition, a significant decrease in ROS generation was also observed in the presence of the extracts tested. The results obtained in this work show that these extracts could be interesting in order to elaborate functional foods and not only for improving their antioxidant properties but also for playing an important role in the treatment of colorectal carcinoma.

Keywords: Pine bark extracts, Antiproliferative effect, Antioxidant activity, Caco-2 cells, Functional foods, ROS

1. Introduction

A wide variety of vegetables are highly appreciated for their nutraceutical potential due to their bioactive components. This makes these vegetables very interesting both for the development of functional foods as well as for their use in pharmacology. Above all, it is important to explore the presence of nutraceutical compounds in inedible vegetables because there is no sense in destroying a food in order to enrich another. Over many years, a great number of plants have been studied in order to identify and isolate efficient biological active components which have antioxidant and anti-proliferative properties in cancer disease. Free radicals are caused by different factors such as oxygen metabolism, radiation, drugs, sunlight, cigarette smoke, dietary fats, certain chemicals, and from contact with environmental pollutants. Free radicals cause cellular damage and play a key role in heart disease, arthritis, cancer, Alzheimer' disease, cataracts and in the hardening of arteries. Antioxidants act as donors of electrons that neutralize Reactive Oxygen Species (ROS) and other free radicals that may otherwise damage DNA and facilitate the formation of tumors (Willcox, Ash, & Catignani, 2004). Nutraceuticals, especially phenolic compounds, are very important bioactive compounds that not only act as antioxidants but also display several functions related to cell differentiation, deactivation of pro-carcinogens, maintenance and reparation of DNA, and other important actions (Shahidi, 2004). Among the phenolic compounds, flavonoids, phenolic acids, stilbenes and tannins, especially condensed tannins (proanthocyanidins), are particularly important. Depending on their structure, flavonoids display possible inhibitory effects on the growth and proliferation of certain malignant cells *in vivo*, and the effects are thought to be either direct, due to their electron and proton donor capacity, or indirect due to their ability to alter the activities of key enzymes in cellular response (Agullo et al., 1997).

Procyanidins are the most abundant polyphenols in plants after lignins, and they may represent up to 50% in barks. Pine is one of the plants with the highest content of procyanidins and they have diverse biomedical applications (Touriño et al., 2005). Within the pine families, *Pinus pinaster*, also known as Maritime Pine, ~~is a species which extends throughout Spain, Portugal, south of France, Italy and Morocco and there are also small populations in Algeria, Malta and the north of Iran. This species~~ has been the most studied and the most used in medicine. From this pine bark, two commercial products such as Pycnogenol® (PYC) and Flavangenol® have been extracted. This latter product is obtained by extracting the bark with hot water. However, PYC is obtained by extracting the bark with water and alcohol, and has long been used to cure open wounds and skin damage and to prevent scurvy (Ohkita, Kiso, & Matsumura, 2011). ~~It has been reported that Flavangenol® has a preventive effect on skin cancer caused by UVB, an antihypertensive effect and a renal disorder suppressing effect as well as an arteriosclerosis suppressing effect (Sugaya, Igarashi, Kojima, Tsubata, & Nagaoka, 2011; Tanida et al., 2009). Nakayama et al. (2015) found that Flavangenol® prevents low density lipoprotein oxidation and regulates monocytic expression of antioxidant enzymes.~~ Pine bark extracts have been also used for the development of functional foods. Thus, Vuorela et al. (2005) added pine bark extract to cooked pork meat and they observed that it was an excellent antioxidant towards protein oxidation. Frontela-Saseta et al. (2011) studied the antioxidant and anti-proliferative effect of fruit juices enriched with PYC in colon carcinoma cells. To do so, they used different fruit juices such as pineapple juice, red fruits juice. In this study, it was found that the addition of PYC to fruit juice increased the content of total phenols, yet this increase did not always correspond with an increase in the antioxidant activity. It was also found that the addition of PYC to pineapple juice produced a higher inhibition of Caco-2 cell growth

compared with the unenriched ~~must~~. *Pinus massoniana* bark extract, ~~which has antioxidant, anticarcinogenic, antimutagenic, antimicrobial and anti-inflammatory effects (Li, Feng, Zhang, & Cui, 2015; Li, Feng, Zhang, Li & Cui, 2016; Monagas, Quintanilla-Lopez, Gomez-Cordoves, Bartolome, & Lebron-Aguilar, 2010),~~ has been used for the production of Songzhen noodles (Zhongjianxing, 2015a) and for chicken feed additives in the production of Songzhen fresh eggs (Zhongjianxing, 2015b).

~~*P. pinaster* has been investigated quite deeply, but there is much less information with regard to other pine species which are of interest and which abound widely in many geographical areas. Yesil-Celiktas et al. (2009) determined the polyphenol compounds and the biological activity of bark extracts from *P. brutia*, *P. sylvestris*, *P. nigra* and *P. pinea*. These samples showed high biological activities and as such, they have a high potential for use in alimentation and pharmaceutical industry. Thus, the aim of this work was to determine whether these pine extracts may inhibit the growth of human colon cancer cell lines (Caco-2) by apoptotic way. Therefore, the present in vitro study aimed to evaluate the anti-proliferative effect and antioxidant activity of extracts from pine bark (*P. pinea*, *P. pinaster and P. halepensis*) on Caco-2 cells and determine the possible mechanism of action~~.

For all the above-mentioned reasons, the possible use of *Pinus* spp. bark, a byproduct of the forestry industry, is interesting as a potential functional ingredient in the food industry, for its antioxidant and biomedical purposes. In this work, we wished to explore the possible use of bark from different *Pinus* species. To this end, the target has been to make an *in vitro* evaluation of the anti-proliferative effect and antioxidant capacity of *P. pinea*, *P. pinaster* and *P. halepensis* extract bark, to determine whether these pine extracts may inhibit the growth of human colon cancer cell lines (Caco-2) by apoptotic way.

2. Material and methods

2.1. Plant materials. Preparation of pine bark extracts and HPLC analysis

This study has been carried out with three pine barks: *P. pinea*, *P. pinaster* and *P. halepensis*. The pine barks used were collected in two different locations in La Rioja (Spain): *P. pinaster* in Villanueva de Cameros (42° 10′ 4″N, 2° 39′ 0″ W, altitude, 900 m), and *P. pinea* and *P. halepensis* in the Sorzano area (42° 20′ 33″ N, 2° 31′ 41″ W, altitude, 722 m). The bark samples were collected in 2015. The specimens were dried and stored at room temperature for further analysis.

Pine bark extract was obtained by Masquelier modified method (Masquelier, 1978). First of all, powder residues from pine bark were removed using a sieve with a pore size of 0.3 mm. Pine bark (50 g) was extracted with boiling water (300 mL) and then cooled down to 20 ºC. After filtration, sodium chloride was added up to saturation and the precipitate formed was removed by filtration. The filtrate was extracted three times with ethyl acetate (10 mL filtrate per 1 mL ethyl acetate). The combined organic extracts were washed with NaCl and dried over Na_2SO_4 anhydrous, filtered and reduced to 1/5 of its volume under vacuum. The extract was then poured into three volumes of chloroform while stirring mechanically. The polyphenols were precipitated and collected by filtration. The light beige powder obtained was stored at -80 ºC. Analysis of the pine extracts composition was performed with a high-pressure liquid chromatograph (Waters Chromatography Div., Milford, MA). Analyses were carried out in gradient with two mobile phases using two 515 pumps: phase A (0.1% phosphoric acid) and phase B (acetonitrile), both from Scharlab (Barcelona, Spain). The flow rate was 1 mL/min with the linear gradient profile that follows: 0–20 min (10 to 22% phase B); 21–40 min (22 to 40% phase B); 41–50 min, (40 to 55% phase B); 51–60 min, (55 to 10% phase B); 61–65 min, equilibration at 10% phase B. The procedure

for different compounds identification was the double coincidence of the UV-visible spectrum at wavelength characteristic of each compound. To do so, a 996 Photodiode Array was used at different wavelengths (200-600 nm). The software employed for chromatographic control was Empower 2.0. An Atlantis dC 18 reverse phase column (150 mm × 4.6 mm i.d., 5 µm particle size) was used. Samples were dissolved in methanol and the injection volume was 10 µL. Fig. 1 shows a chromatogram.

Standard solutions of catechin, procyanidin A2, procyanidin B1, procyanidin B2 and taxifolin (taxifolin: purity≥90%, Sigma-Aldrich, Madrid, Spain; catechin: purity≥99%, Sigma-Aldrich; procyanidin B1 and B2: purity≥90% Extrasynthese, Lyon, France; procyanidin A2: purity≥99% Extrasynthese) were prepared in methanol (100 ppm), which was stable in the dark and at 0 °C. To establish calibration curves, five diluted solutions were prepared from the standard primary solution (0.75, 0.5, 0.25, 0.10 and 0.05 ppm) obtaining a linear detector response ($R^2 \geq 0.9913$).

2.2. Antioxidant activity of pine bark extract

Total antioxidant activity was measured according to ABTS, 2,2'-azino-bis-(3-ethylbenzoithiazolone-6-sulphonic acid), method described by Cano, Hernández-Ruiz, García-Canovas, Acosta, and Aranao (1998). To do so, a Jasco Spectrophotometer V-630 (Washington, USA) was used. The calibration curve was performed with six ascorbic acid solutions at concentrations between 90 µM and 300 µM. The total antioxidant activity was expressed as mg of ascorbic acid per g of pine bark extract.

2.3. Cell culture, cell treatment and determination of cytotoxicity

The biological activity of bark extracts have been tested against human colon cancer cell line Caco-2 clones PD7 (from early passage and heterogeneous) and TC7 (from late passage and homogeneous). This cell line, derived from human colon

adenocarcinoma, is one of the most regularly used for intestinal drug studies. It undergoes spontaneous enterocytes differentiation when cultured over confluence for 21 days to become polarized cells expressing apical and basolateral surfaces with well-established tight junctions. This cell line was provided by Dr. Edith Brot-Laroche (Université Pierre et Marie Curie-Paris 6, UMR S 872, Les Cordeliers, France).

Maintenance of the cells was completed at a constant temperature of 37 °C and in CO_2 atmosphere (5%). The cells were grown in Dulbecco's Modified Eagles medium (Gibco Invitrogen, Paisley, UK) supplemented with non-essential amino acids (1%), fetal bovine serum (20%), 1000 µg/mL streptomycin (1%), 1000 U/mL penicillin (1%) and 250 U/mL amphotericin (1%). The cells were passaged enzymatically with 0.25% trypsin-1 mM EDTA and cultured on plastic flasks (25 or 75 cm^2) at a density of $2x10^4$ cells/cm^2. The medium of culture was changed every 2 days. The confluence of the cells (80%) was determined by optical microscopy. Experiments were performed 24 hours post-seeding to prevent cell differentiation (García-Moreno, Gascón, Rodríguez-Yoldi, Cerrada, & Laguna, 2013). In order to carry out the cell treatment and the determination of cytotoxicity, pine bark extracts (*P. pinea, P. pinaster* and *P. halepensis*) were diluted in the medium to the required concentration (varying from 0 to 1000 mg/L) with an exposure time of 72 h. The same protocol is followed for the treatment of cells with procyanidins B1 and B2 at different concentrations (5.29 to 42.46 µg/g). The cell survival was measured using the Sulforhodamine B (SRB) assay (Skehan et al., 1990). At the end, the results were obtained by measuring absorbance with a multiwell spectrophotometer (Biotek Synergy HT, Vermont, USA) following the protocol previously described in Jiménez et al. (2016). The effect on cell growth was expressed as % control.

2.4. Cell cycle analysis and apoptosis measurements

The human Caco-2 cells (PD7 and TC7 clones) were exposed to 20 mg/L of the three different pine bark extracts for 72 h; then they were collected and stained with Annexin V-FITC. This concentration was selected as the closest to the around IC50 viability. Propidium iodide (PI) stained cells were analysed for DNA content in a BD FACSArray (New Jersey, USA) and the percentage of cells in cycle phases was determined using MODIFIT 3.0 verity software following the protocol of Jiménez et al. (2016).

In order to determine basal levels of apoptosis, necrosis and cell death, untreated cells were used as negative control. After incubation, cells were analysed by flow cytometry within 1 h. The signal intensity was measured using a BD FACSAria (New Jersey, USA) and analysed using the BD FASCDiva software following the protocol previously described in Jiménez et al. (2016).

2.5. Determination of MMP, cytochrome c and caspase 3 by flow cytometry

Caco-2 cells lines PD7 and TC7 clones were cultured in 75 cm^2 flask at a density of 500.000 cells per flask and incubated 24 h under standard cell culture conditions. For treatment, pine bark extracts at concentration of 20 mg/L were added to each flask and incubated 72 h. After treatment cells were washed twice with temperate PBS and then resuspended in temperate PBS at a concentration 1 x 10^6 cells/mL. 5 µL of 10 µM cationic dye 1,1',3,3,3'-hexamethylindodicarbo-cyanine iodide (DiIC1) were added to each sample and cells were incubated 15 min at 37 ºC, 5% CO_2. After an incubation period, 400 µL PBS were added to each tube and fluorescence was analyzed by flow cytometry using a FACSARRAY BD equipped with an argon ion laser. Excitation and emission settings were 633 and 658 nm, respectively (Sánchez-de-Diego et al., 2017).

The cytochrome c release was determined as described by Christensen et al. (2013). Briefly, cells were resuspended in 100 µL ice-cold permeabilization buffer (100 mM KCl, 50 µg/mL digitonin in PBS) and incubated for 5 minutes. Then, 100 µL of 4% paraformaldehyde in PBS was added to permeabilised cells and the mixture was centrifuged 5 min at 500g at 4 ºC. Supernadant was removed and pellet was resuspended in 4% paraformaldehyde and incubated 20 min at room temperature (RT). After incubation, cells were washed three times in 200 µL PBS. The obtained pellet was resuspended in 200 µL permeabilization buffer (0.05% Saponin, 3% BSA in PBS) and incubated for 15 min at RT. Then, 2 µL of anti-cytochrome antibody (Novus (7H8.2C12)[PE]) were added and the mix was incubated for 1 hour at RT. After incubation, cells were centrifuged 5 min at 500g at RT and washed twice with PBS. Finally, cells were resuspended in 400 µL PBS. Fluorescence was analyzed by flow cytometry using a FACSARRAY BD equipped with an argon ion laser. Excitation and emission settings were 488 and 575 nm, respectively.

For the determination of caspase 3, once treated the cells with 20 mg/L of pine extracts, the cells were fixed in 0.01% formaldehyde for 15 min and centrifuged for 5 min at 300g and RT. Then, for cell membranes disruption, the pellet was suspended in 100 µL of 0.5% v/v in PBS digitonin solution and incubated for 15 min in the dark at RT. After incubation, cells were washed with 2 mL of PBS containing 0.1% diginonin and centrifuged at 300g for 5 min. Supernatant was discarded and pellet was resuspended in 200 µL of PBS containing 0.1% diginonin. The antibody (2 µL) anti-Caspase 3 (Novus, Clone C92-605) was added to each sample and the mix was incubated for 1 hour at RT. After incubation, cells were centrifuged 5 min at 500g at RT and washed twice with PBS. Finally, cells were resuspended in 400 µL PBS. Fluorescence was analyzed by flow cytometry using a FACSARRAY BD equipped

with an argon ion laser. Excitation and emission settings were 494 and 520 nm. respectively.

2.6. Intracellular levels of ROS

The intracellular level of ROS was assessed using the DCF assay (Ruiz-Leal, & George, 2004). Caco-2 cells were seeded in 96-well plates at a density of 4×10^3 cells/well. Before oxidative stress induction, cells were cultured for 24 h, and then incubated (72 h) with serum-free culture media with two different concentrations of the different pine extracts (20 or 1000 mg/mL). After treatment, the cells followed the protocol described in Jiménez et al. (2016). ROS intracellular level is expressed as fluorescence arbitrary units, expressed as a percentage from cells fluorescence with H_2O_2 without preincubation in pine extracts.

2.7. Statistical analysis

Statistical analysis and the graphics were performed using the GraphPad Prism Version 5.02 software. Results are expressed as means ± SEM. Means were compared using the analysis of variance (ANOVA) and significant differences ($p < 0.05$) were determined using a Bonferroni's Multiple Comparison Test.

3. Results and discussion

3.1. Antioxidants in pine bark extracts

The most abundant antioxidants compounds in the pine samples analyzed in this work were taxifolin and catechin (Table 1). Iravani and Zolfaghari (2014) studied the composition of *P. pinaster* and *P. eldarica* barks coming from Iran, and they also found that the main substances identified in these species of pine were taxifolin and catechin. ~~The amount of catechin both in *P. pinea* as in *P. pinaster* was very similar, at around~~

~~100 µg/g of pine bark while *P. halepensis* showed a lower concentration of this compound (46 µg/g of pine bark) (Table 1).~~ The concentration of catechin in *P. pinea* and *P. pinaster* bark extract was the same, at around 100 µg/g pine bark, while *P. halepensis* showed a lower content of this compound, 46 µg/g pine bark (Table 1). Yesil-Celiktas et al. (2009) determined the polyphenol composition of different pines coming from Turkey (*P. brutia*, *P. pinea*, *P. sylvestris* and *P. nigra*), and found that the concentration of catechin varied between 17.8 mg/g of pine bark in *P. sylvestris* and 70.4 mg/g of pine bark in *P. brutia*. The concentration of catechin in *P. pinea* was 35.8 mg/g of pine bark. Catechin ~~behaves as a powerful antioxidant and free radical scavenger and~~ is able to reduce platelet aggregation and to inhibit the growth of human cancer cell lines (Putter et al., 1999). Besides, this flavan-3-ol may act as a cardioprotective agent because it protects human low density lipoprotein (LDL) against oxidation (Yilmaz, & Toledo, 2004). Catechin has two pharmacophore groups, a catechol and a resorcinol group. The antioxidant action of this compound is very interesting due to its ability to scavenge free radicals by electron-transfer processes. Catechol's oxidation mechanism proceeds in sequential steps, related to the catechol and resorcinol groups and the oxidation is pH dependent (Janeiro, & Oliveira Brett, 2004). The content of taxifolin was slightly higher in *P. pinaster* bark (128 µg/g) than in *P. pinea* bark (112 µg/g). *P. halepensis* showed the lowest concentration of this compound (83 µg/g). Yesil-Celiktas et al. (2009) reported concentrations of this compound between 3 mg/g and 186 mg/g of pine bark. Romani et al. (2006) found 33.1 mg/g of taxifolin in a commercial pine bark extract from *P. maritima*. Taxifolin, also known as dihydroquercetin, is a flavanonol derivative of flavonoids which plays a special role in the circulatory system because of its significant anti-inflammatory properties (Kim, Choi, Lee, & Lee, 2008) and antioxidant activity (Liang et al., 2013).

Taxifolin also reduces the formation of cancer cells (Manigandan, Jayaraj, & Elangovan, 2014).

Procyanidins are a type of flavonoids consisting of oligomers of flavan-3-ols. Most of the activities of procyanidins largely depend on their structure and, particularly, on their degree of polymerization (Touriño et al., 2005). Procyanidin B2 has been highlighted as one of the most active molecules within the procyanidins (Yang et al., 2014). Similarly, procyanidin B2 has been associated with anti-inflammatory properties (Martínez-Micaelo, González-Abuin, Pinent, Ardévol, & Blay, 2015), cardioprotective and neuroprotective effects (Kopustinskiene et al., 2015; Wu et al., 2015), anti-proliferative activity (Avelar, & Gouvêa, 2012). Procyanidin B1 has been much less studied, although it seems that it has anti-proliferative (Okamoto et al., 2014) and neuro-protective actions (Kanno et al., 2015). In *P. pinea* bark extract the amount of procyanidin B1 was 14 μg/g of pine bark while the content of this compound in *P. pinaster* and *P. halepensis* was lower (5.3 and 6.9 μg/g of pine bark, respectively). In the case of procyanidin B2 just the opposite occurred, *P. pinaster* had the highest concentration of this flavonoid (Table 1). *P. halepensis* was the only one that showed procyanidin A2 in its bark. Disparity between the procyanidin content in bark of the different *Pinus* species studied can arise from natural variability such as genotype, differences in growing and harvesting conditions, climate, soil type, etc. (Hellström et al., 2009). ~~Total antioxidant capacity was similar in *P. pinea* and *P. pinaster* and somewhat lower in *P. halepensis* (Table 1)~~. Total antioxidant capacity of *P. pinaster* was higher than that of *P. pinea*, and *P. halepensis* had the lowest antioxidant capacity (Table 1).

3.2. Antiproliferative activity of pine bark extracts on Caco-2 cells

~~The biological activity of *P. pinea*, *P. pinaster* and *P. halepensis* bark extracts have been tested against human colon cancer cell line Caco-2 clones PD7 (from early passage and heterogeneous) and TC7 (from late passage and homogeneous). This cell line, derived from human colon adenocarcinoma, is one of the most regularly used for intestinal drug studies. It undergoes spontaneous enterocytes differentiation when cultured over confluence for 21 days to become polarized cells expressing apical and basolateral surfaces with well-established tight junctions. Experiments in undifferentiated cells were performed 24 hours post-seeding.~~ Exposure of Caco-2 cell lines to increasing concentrations (0-1000 mg/L) of all the bark pine extracts exhibited, after a continuous exposure during a 72-hour period, a dose-dependent growth inhibitory effect. The results are expressed in terms of % viability with respect to control (Figs. 2A and 2B), using the well-established SRB assay (see Material and Methods for details).

3.3. Effect of pine bark extracts on apoptosis in colon cancer cells

Since pine extracts treatment proved true in reducing cell viability, it was decided to determine what type of cell death occurred. To address this doubt, flow cytometry analyses over 72 hours were performed using the Annexin-V/PI double-staining assay, which are well-established biomarkers of cell death (Li et al., 2014).

Results showed that after 72 hours, the treatment with different pine extracts at 20 mg/L, produced a higher apoptosis in both cancer cell lines (PD7 and TC7) than in cells without treatment. It should be noted that *P. pinaster* extract was the extract that induced most early and late apoptosis (Table 2, Fig. 3). In summary, the results obtained show that all pine extracts were able to induce cell death by activating apoptotic

pathways, thereby reducing their ability to non-selectively react with biological targets to cause necrosis and its related side effects.

Afterwards, the alteration of MMP was analyzed. During apoptosis, an uncontrolled production of oxygen radicals, as well as a disruption of the pro-apoptotic vs. anti-apoptotic protein balance produce changes in MMP and stimulate cytochrome c release from mitochondria and finally caspase 3 activation (Kuwana et al., 2002). Then, the changes in MMP were analyzed by using the cationic dye DilC1 and flow cytometry. MMP changes in cells treated with pine bark extracts were found compared with control cells (Fig. 4). The extracts could possibly interact with different molecules inside the cell, and also inside the mitochondria, disrupting the energy metabolism that would lead to a reduction of MMP. Changes in MMP are tightly related to apoptosis and can produce the redistribution of cytochrome c from the inner mitochondria to cytoplasm (Gottlieb, Armour, Harris, & Thompson, 2003). We used flow cytometry in order to determine whether our pine bark extracts were able to induce mitochondrial permeabilization and cytochrome c release. Results showed a greater cytochrome c release in treated cells than in the untreated ones (Fig. 5). Once cytochrome c is release to the cytoplasm, it could activate different proteins of the intrinsic apoptosis pathway such as the effector caspase-3 (Riedl, & Shi, 2004). Therefore, we analyzed the levels of this caspase in cells treated with pine extracts compared to control (Fig. 6). ~~It was observed an increase in active caspase-3, compatible with the induction of apoptosis in Caco-2 cells.~~ Based on our results, we can say that the increase in the activity of caspase 3 observed is compatible with the induction of apoptosis in Caco-2 cells. Therefore, ~~our results suggest~~ it may be proposed that the pine bark extracts induce changes in mitochondrial permeability and triggers cytochrome c release. These alterations

promote the activation of the caspase cascade that culminates in the execution of apoptosis.

3.4. Effect of pine bark extracts on Caco-2 cell cycle

It has been reported that the normal progression of cell cycle could be affected by DNA damage events; in particular cells with DNA damage arrest their cell cycle in G_2/M or S phase (Abid-Essefi et al., 2003). If DNA damage is not repaired, cells can undergo apoptosis or necrosis. In this study, the effects of the pine extracts on cell-cycle progression in both cancer cell lines after 72 hours of treatment were analyzed. Cell-cycle analysis was performed by using flow cytometry to assess the DNA content of cells stained with PI. Studies of Hu, Zhang, Qiu, Yu, and Lin 2010 revealed that anti-cancer agents arrest the cell cycle at the G_0/G_1, S or G_2/M phase and then induce apoptotic cell death. The cell cycle arrest has become an appreciated target for management and treatment of tumor cells with cytotoxic agents (Schwartz, & Shah, 2005). The fluorescence intensity of sub G_0 cell fraction represents the apoptotic cell population (Jain et al., 2013).

Cell cycle analysis of PD7 and TC7 cells treated with the different extracts of pine is represented in Fig. 7. Results showed an increase in the S phase with a decrease in the G_1 and G_2 involving an S-phase arrest. In addition, in the case of *P. pinaster*, there was a disruption of the cell cycle. This fact could be in accordance with the highest apoptosis observed for this extract. Therefore, pine bark extracts altered the cell cycle in Caco-2 cells. This finding could support the induction of apoptosis confirmed by MMP increase with cytochrome c release to cytoplasm and caspase 3 activation.

3.5. Effect of pine bark extracts on ROS intracellular levels

The oxidative stress imposed by ROS plays an important role in many chronic, degenerative diseases and cancer. Natural antioxidants obtained from plants and vegetables are generally needed to counteract the damage of ROS to cells. Thus, low-to-moderate levels of ROS are essential for cellular proliferation, differentiation, and survival (Trachootham, Alexandre, & Huang, 2009). The changes in the redox status of Caco-2 cells in response to pine bark extracts were determined. Cells were exposed to 20 and 1000 mg/L of pine extracts for 72 hours and the intracellular levels of ROS were determined by DCF assay. After pine extracts exposure of Caco-2 cells, low levels of oxidizing species were obtained when compared to the basal rate, ~~being slightly higher the antioxidant power of *P. pinaster* extract~~ being *P. pinaster* the one that presented a greater antioxidant power (Figs. 8A and 8B). These disturbances in redox balance could be a cause or a consequence of the mitochondrial alterations.

3.6. Effect of procyanidins on cellular viability

Proanthocyanidins, which include both procyanidins and prodelphinidins, are a particular interesting type of flavonoids. They are powerful free radical scavengers, efficient antioxidants, and anti-proliferative and anti-inflammatory agents (Cos et al., 2004). The main difficulty in studies on procyanidins is probably that of obtaining them in an individual molecular form. Therefore, in order to study their structures and properties, mixtures, more or less polymerized, are often employed (Guyot, Marnet, & Drilleau, 2001). Moreover, synergistic effects of active mixtures make plant extracts more interesting than pure compounds for pharmacological applications. *P. pinaster* extract had the most antiproliferative and antioxidant action of the three pine barks extracts studied in this work. In addition, this pine is the one with the highest procyanidin B2 content. For this reason, it was felt to be interesting to study the effect of procyanindins on cellular viability in order to determine if their content, in these pine

bark extracts, was related to the effect previously observed. We assayed the procyanidins B1 and B2 both separately and together in a range of concentrations similar to those found in the extracts (Table 1). The results showed a lower antiproliferative effect than that observed in the treatment of the cells with the different pine extracts (Table 3). This fact agrees with that observed by Packer, Rimbach, and Virgili (1999) with PYC, a standardized extract from *P. pinaster* bark composed of a mixture of flavonoids, mainly procyanidins and phenolic acids. PYC displayed greater biologic effects as a mixture than its purified components did individually, indicating that the components could interact synergistically without ruling out perhaps a greater effect of procyanidin B2.

4. Conclusion

Our results indicated that pine bark extracts (*P. pinea*, *P. pinaster* and *P. halepensis*) have important antioxidants such as taxifolin, catechin, procyanidin B1 (especially in *P. pinea*), procyanidin B2 (especially in *P. pinaster*). Additionally, procyanidin A2 was present only in *Pinus halepensis*. With regard to the action of bark extracts from different pine species on Caco-2 cells it has been observed that they produced a cytotoxic effect that could be related to disturbances in redox balance. This could even produce cell damage, as well as cell cycle disruption and intrinsic apoptosis induction by involving changes in MMP, cytochrome c release and caspase 3 activation in Caco-2 cells. In addition, procyanidins and other components of pine bark extracts could act synergistically to produce these effects. *P. pinaster* was the one with the highest biological activity and the one with the highest amount of procyanidin B2 which would indicate that this component plays an important role. Therefore the results of this work indicate that pine bark extracts could be used as ingredients in the development of

functional foods, constituting a promising and innovative alternative for this natural product.

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Conflict of interest

No conflict of interest

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FIGURE CAPTIONS

Fig. 1. Chromatogram of a *P. halepensis* bark extract. Peaks: (1) Procyanidin B1; (2) Catechin; (3) Procyanidin B2; (4) Taxifolin; (5) Procyanidin A2.

Fig. 2. Caco-2 cells viability (%) with different concentrations (0-20-40-62.5-125-250-500-1000 mg/L) of *P. pinea*, *P. pinaster* and *P. halepensis* after 72 h to exposure. Results for PD7 cells are in column A and in column B those for TC7 cells. Values are means ± SEM of three independent experiments, each performed with six determinations. *$p < 0.05$ compared with control (without treatment).

Fig. 3. Quantitative flow cytometry analyses using PI uptake and Annexin V staining in PD7 (A) and TC7 (B) colon cancer cells treated with 20 mg/L of pine bark extracts (*P. pinea* -1-, *P. pinaster* -2- and *P. halepensis* -3-) after 72h. In control (C), the cells are without treatment. Experiments were performed in triplicate.

Fig. 4. Quantification by flow cytometry of % cells with MMP changes in Caco-2/PD7 and Caco-2/TC7 cells treated with 20 mg/L of *P. pinea* (1), *P. pinaster* (2) and *P. halepensis* (3) extracts after 72 h. Experiments were performed in triplicate. *$p < 0.05$ compared with control (without treatment).

Fig. 5. Quantification of mitochondrial cytochrome c by flow cytometry in Caco-2/PD7 and Caco-2/TC7 cells treated with 20 mg/L of *P. pinea* (1), *P. pinaster* (2) and *P. halepensis* (3) extracts after 72 h. Experiments were performed in triplicate. *$p < 0.05$ compared with control (without treatment).

Fig. 6. Quantification of % cells with caspase 3 active by flow cytometry in Caco-2/PD7 and Caco-2/TC7 cells treated with 20 mg/L of *P. pinea* (1), *P. pinaster* (2) and *P.*

halepensis (3) extracts after 72 h. Experiments were performed in triplicate. *p < 0.05 compared with control (without treatment).

Fig. 7. Cell-cycle analysis after treatment with 20 mg/L of different pine bark extracts. *P. pinea* (1), *P. pinaster* (2) and *P. halepensis* (3) after 72 h exposure. In control (C), the cells are without treatment. Cell cycle and DNA fragmentation were determined by PI staining. Percentages of G_1, S and G_2-phase are shown when possible. Experiments were performed in triplicate.

Fig. 8. Effect of pine bark extracts on ROS intracellular levels in PD7 (A) and TC7 (B) Caco-2 cells. Caco-2 cells were treated at 20 and 1000 mg/L for 72 h with extracts of *P. pinea, P. pinaster* or *P. halepensis*. % ROS levels comparing the cells treated for 20 min with 500 μM H_2O_2 and pine extracts, to positive control (only with H_2O_2).Values are means ± SEM of three independent experiments, each performed with six determinations. *p < 0.05 compared with positive control.

Table 1

Antioxidant composition and antioxidant activity of pine bark extracts (n=6)

Pine sample	Catechin[a]	Taxifolin[a]	Procyanidin A2[a]	Procyanidin B1[a]	Procyanidin B2[a]	Antioxidant Activity[b]
P. pinea	104 ± 6	112 ± 7	nd	14 ± 2	28.7 ± 0.6	670 ± 10
P. pinaster	102 ± 1	128 ± 4	nd	5.3 ± 0.3	42 ± 1	724 ± 3
P. halepensis	46 ± 6	83 ± 3	22 ± 1	6.9 ± 0.1	10.9 ± 0.1	452 ± 3

[a] Results expressed in μg of antioxidant/g of pine bark

[b] Results expressed in mg of vitamin C/g of pine bark

Table 2

Summary of PD7 and TC7 colon cancer cells treated with 20 mg/L of *P. pinea* (1), *P. pinaster* (2) and *P. halepensis* (3) extracts after 72 h. In control (C), the cells are without treatment.

	Extracts	Live (%)	Early apoptic (%)	Late apoptic (%)	Necrotic (%)
PD7	C	85.2	8.4	4.8	1.6
	1	65.2	22.4	11.0	1.4
	2	14.7	39.6	34.2	11.6
	3	62.2	16.2	20.3	1.2
TC7	C	93.7	2.7	3.0	0.6
	1	63.1	22.3	12.9	1.6
	2	19.2	26.6	46.1	8.1
	3	69.6	16.1	12.3	2.0

Table 3

PD7 and TC7 Caco-2 cells viability (%) treated with different concentrations of procyanidins B1 and B2: 5.29 (a), 14.17 (b), 28.69 (c), 42.46 (d) μg/g of bark after 72 h to exposure. Values are means ± SEM of three independent experiments, each performed with six determinations.

Extracts	PD7 cells	TC7 cells
C	100	100
a-B1	77 ± 4*	78 ± 2*
b-B1	75 ± 4*	80 ± 4*
c-B1	74 ± 3*	76 ± 4*
d-B1	65 ± 5*	69 ± 5*
a-B2	74± 2*	76 ± 3*
b-B2	66 ± 3*	70 ± 4*
c-B2	66 ± 3*	69± 4*
d-B2	69 ± 4*	64 ± 5*
b-B1+cB2	71 ± 3*	66 ± 7*
a-B1+dB2	64 ± 4*#	55 ± 9*#

*$p < 0.05$ compared with control (without treatment)

# $p < 0.05$ compared with a-B1 and a-B2

Figure

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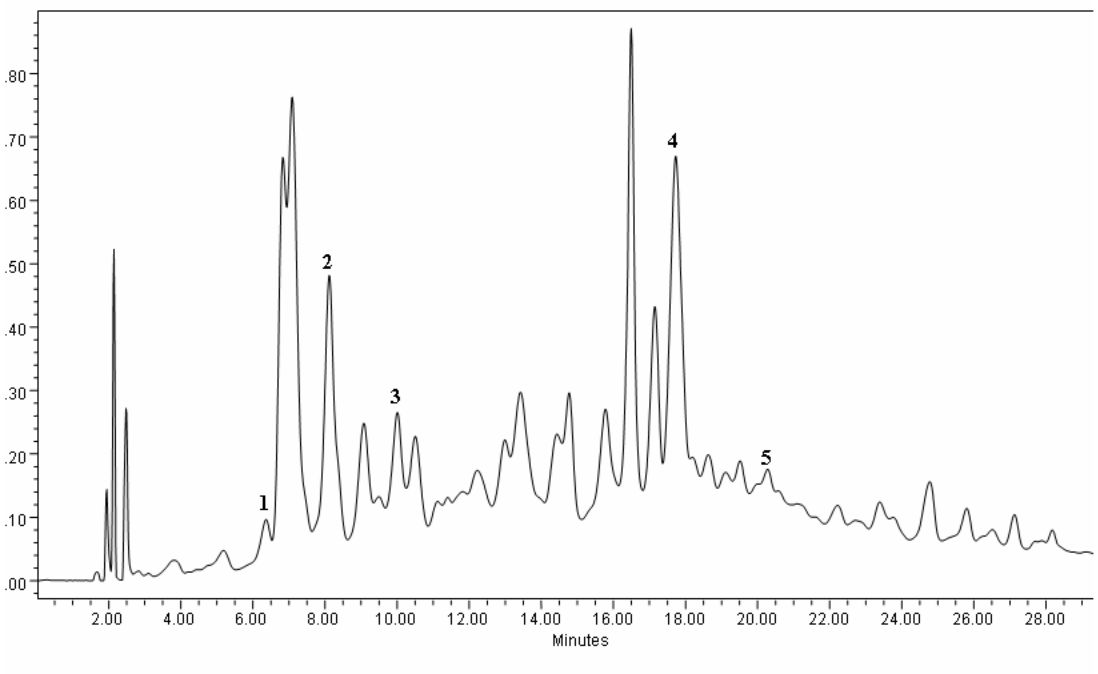

Figure 1

Figure

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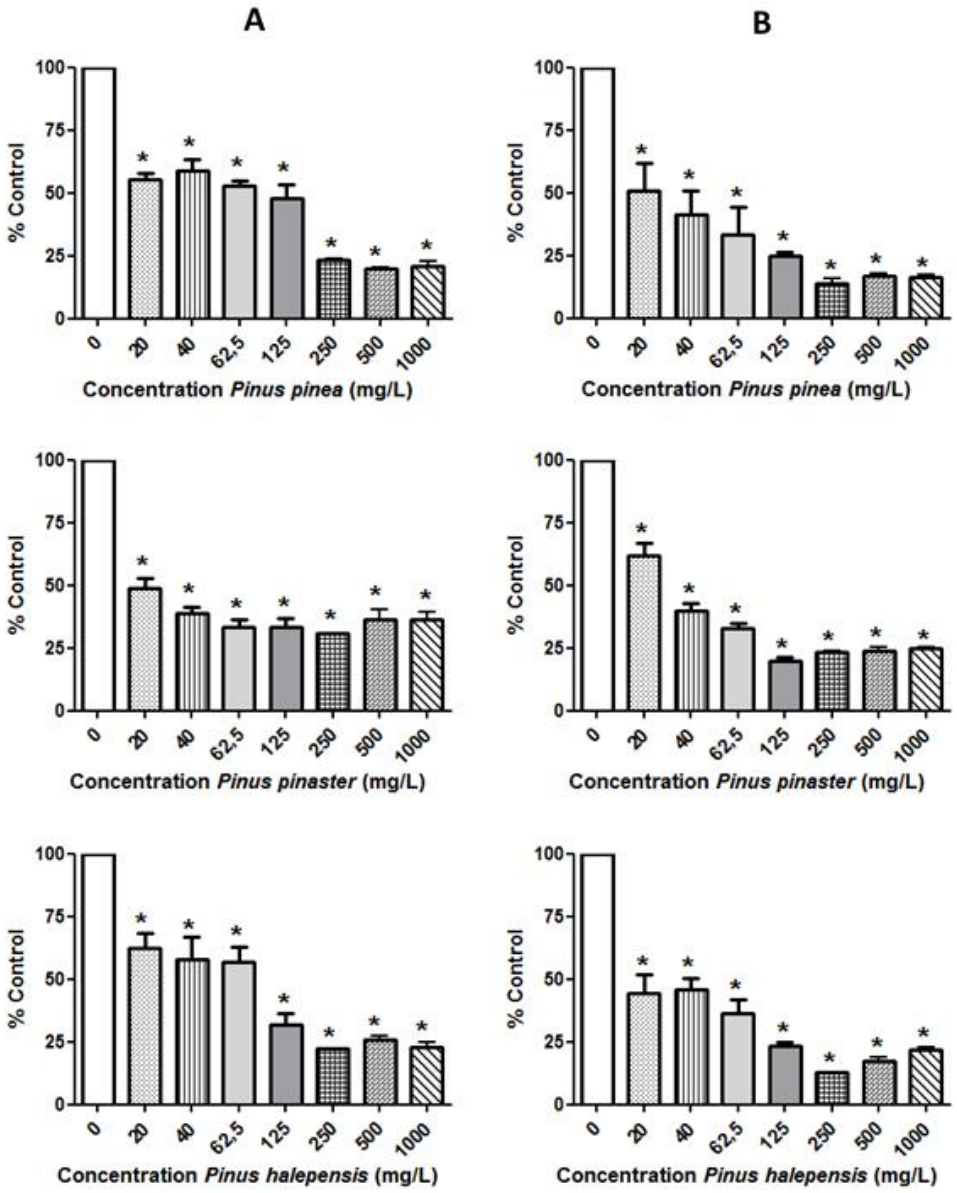

Figure 2

Figure

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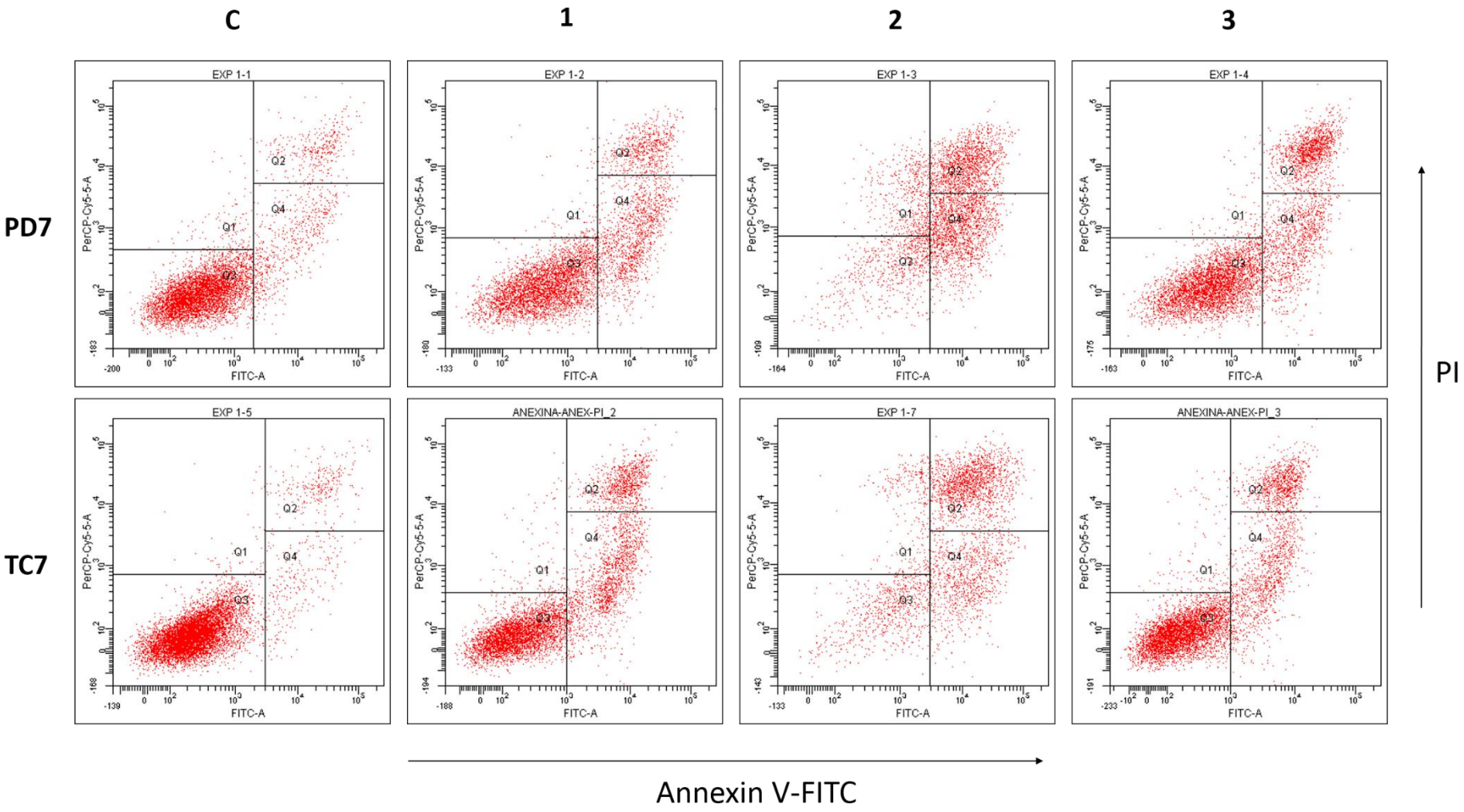

Figure 3

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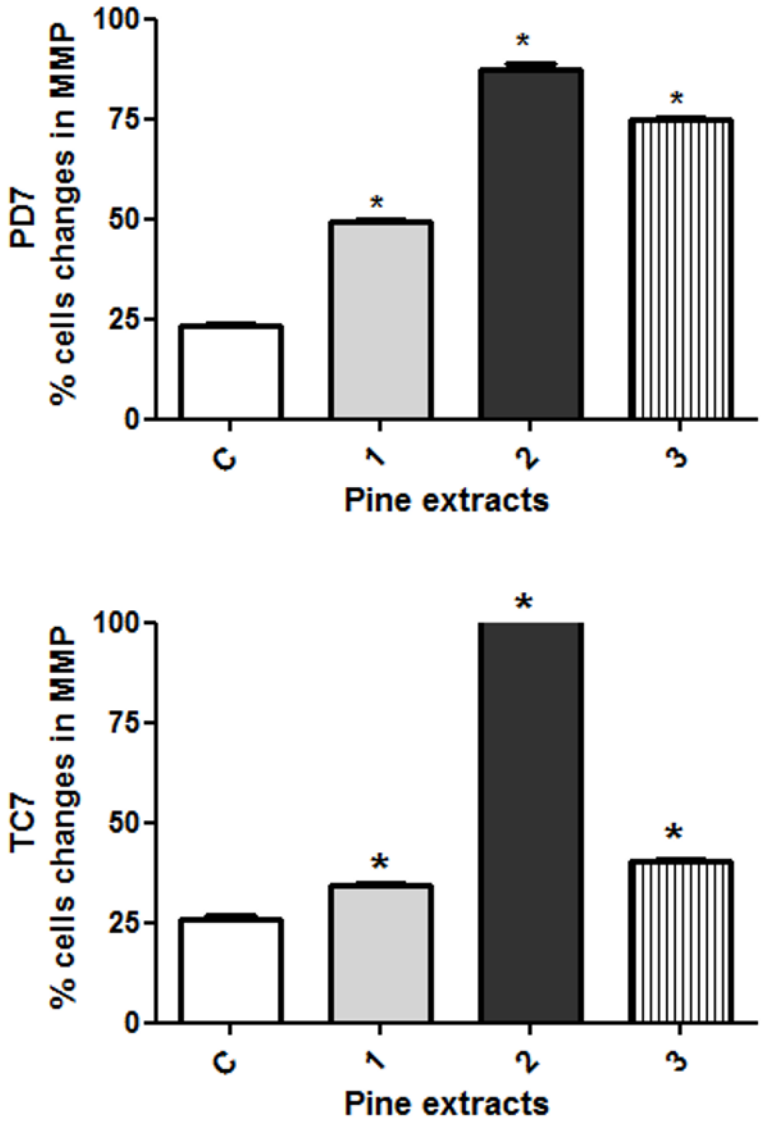

Figure 4

Figure

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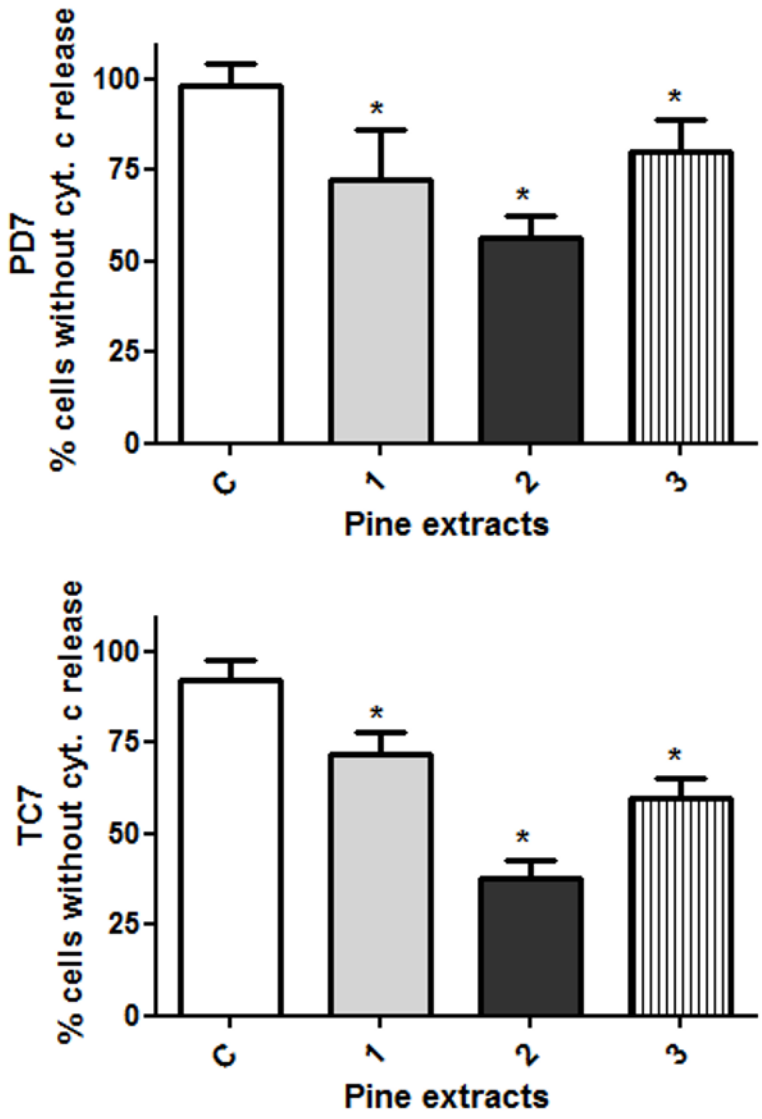

Figure 5

Figure

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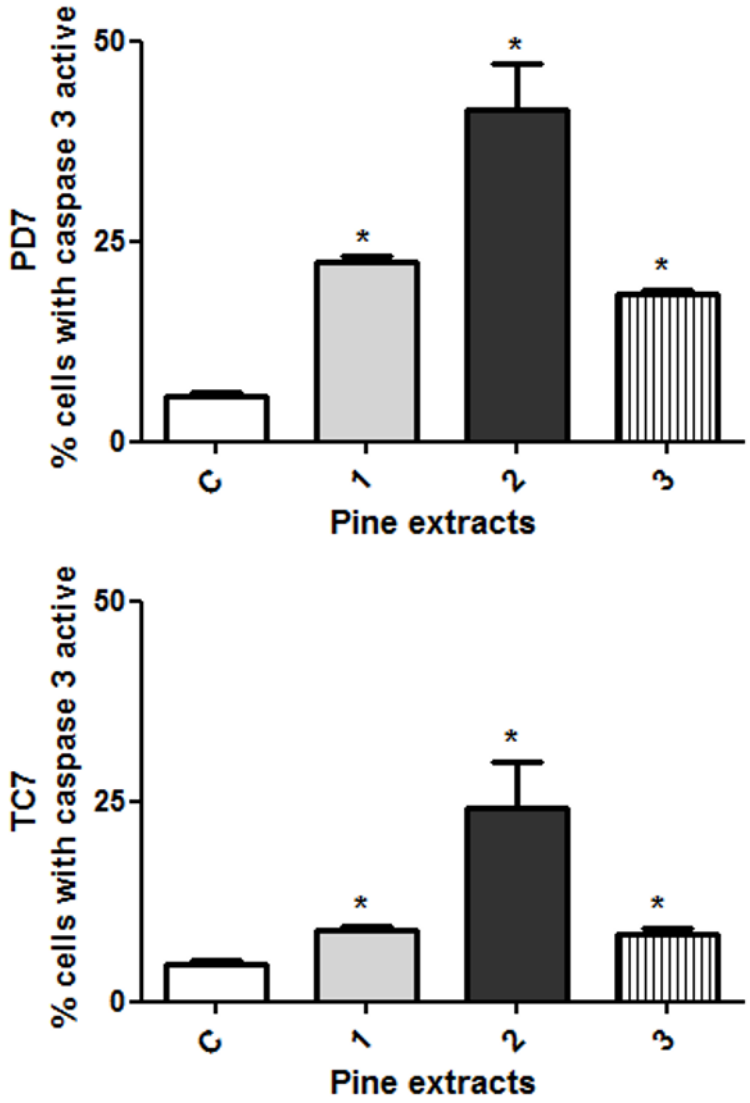

Figure 6

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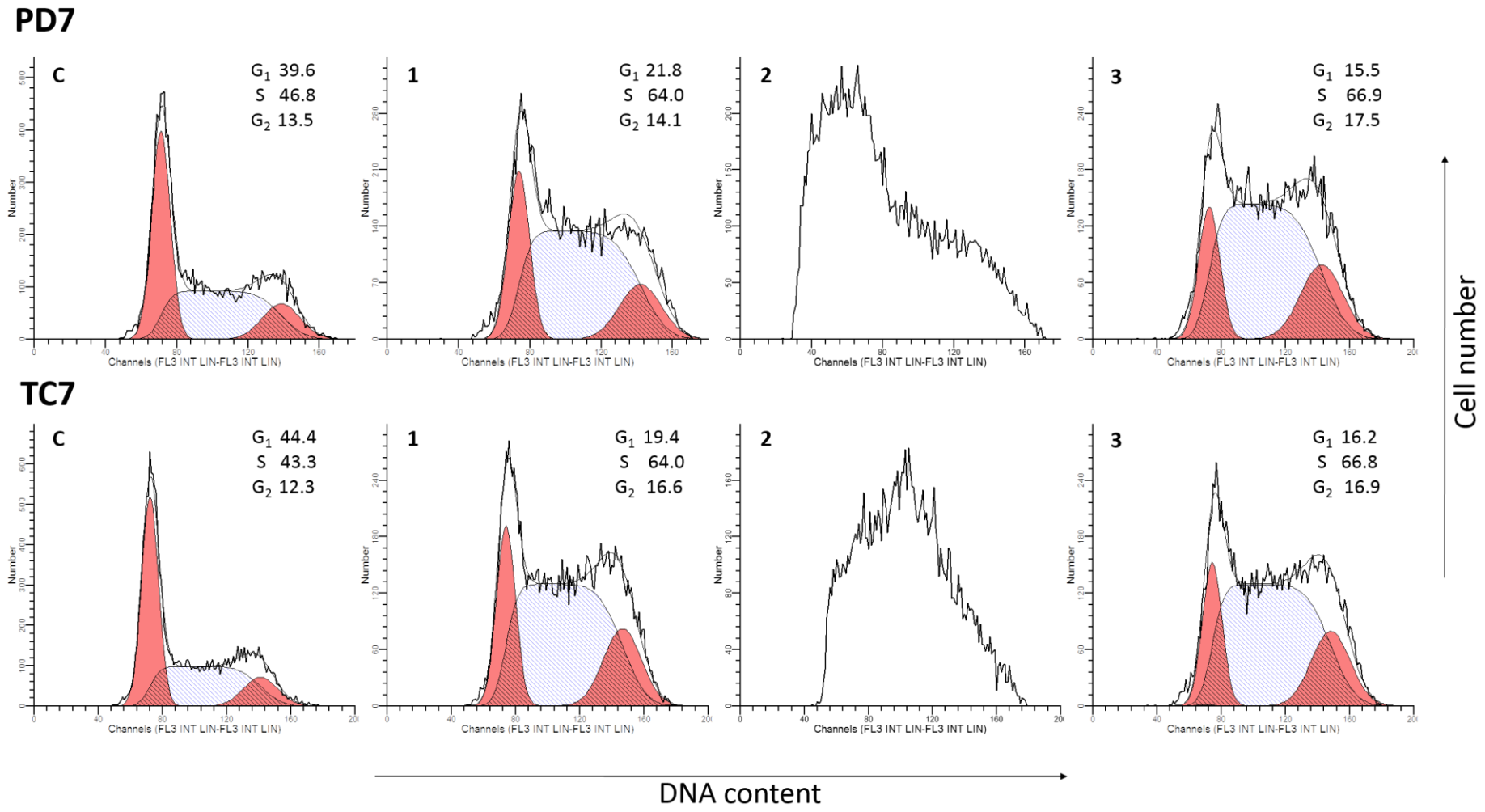

Figure 7

Figure
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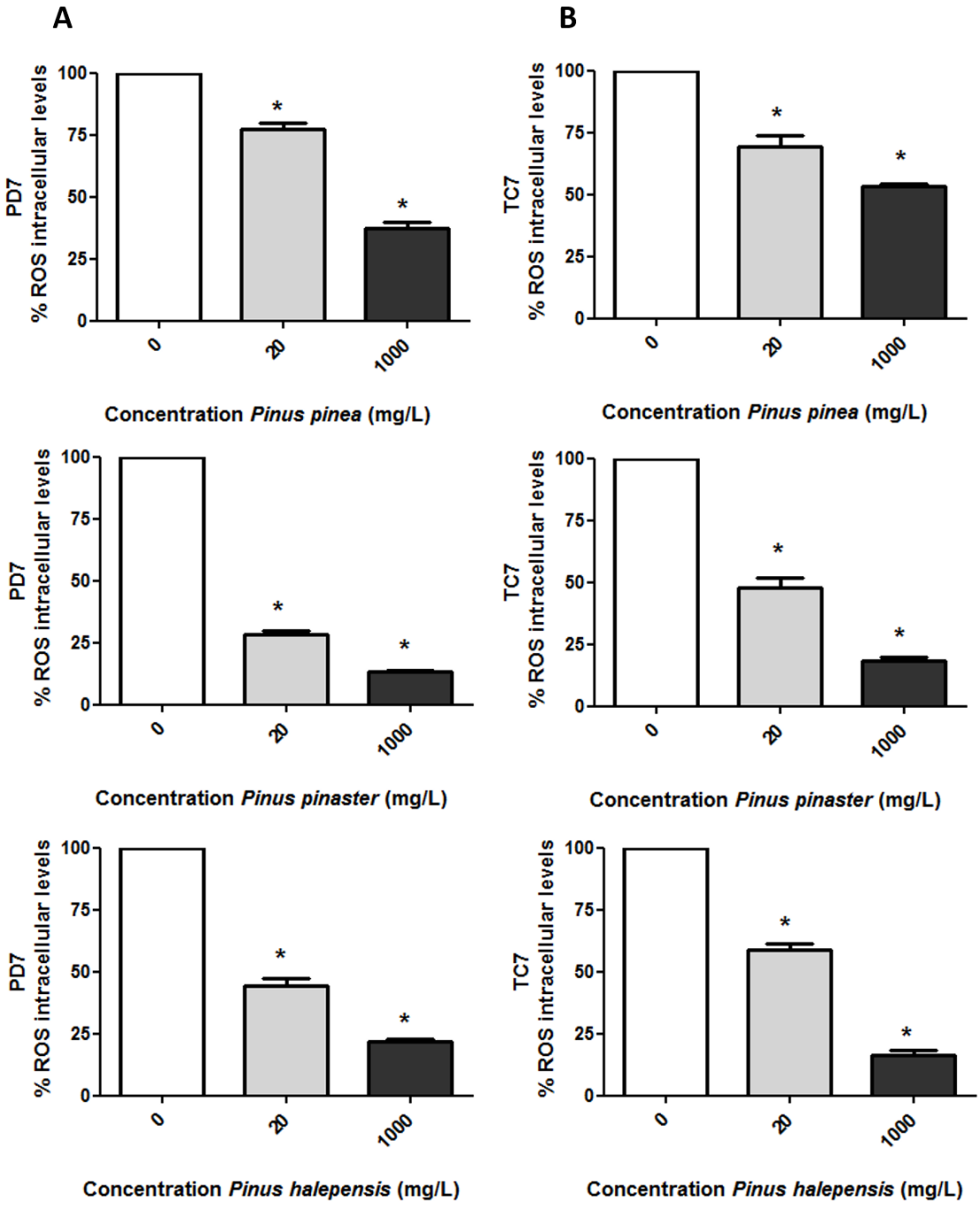
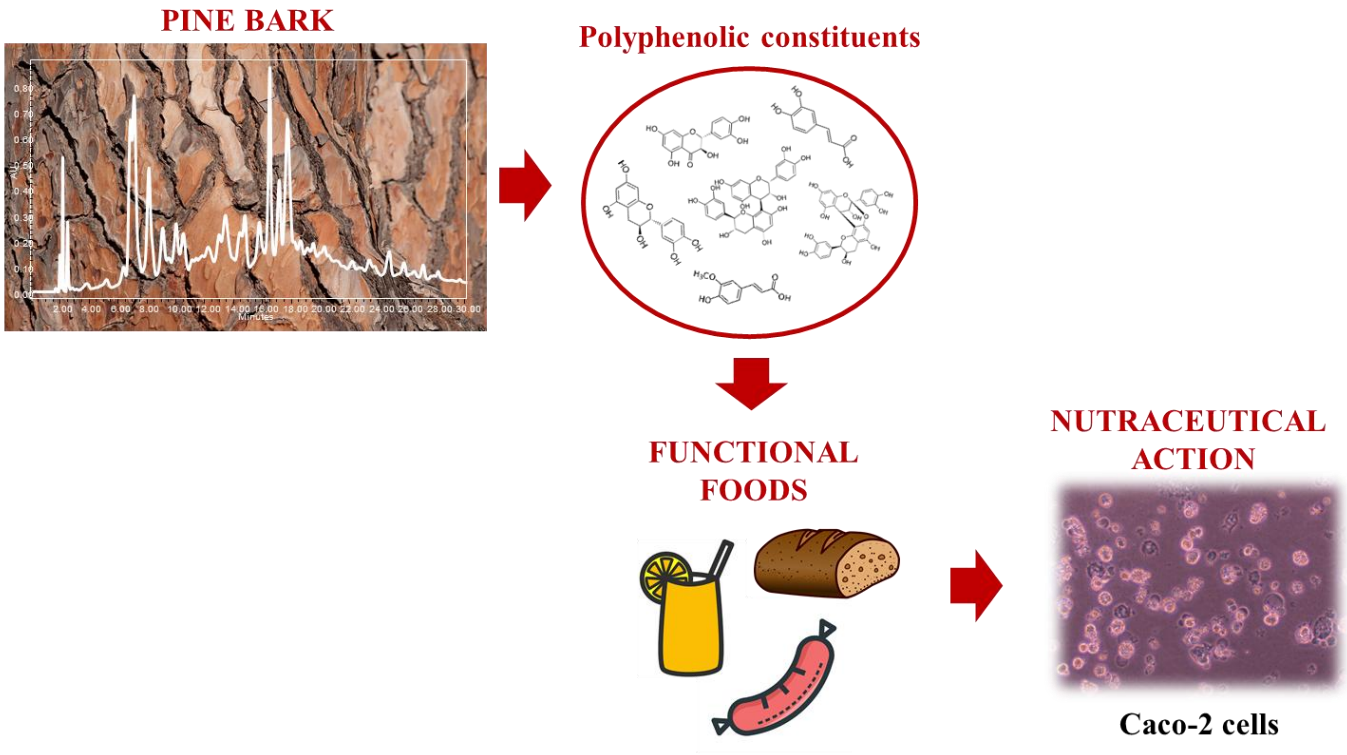


Figure 8

*Graphical Abstract



Conflict of interest

No conflict of interest

Ethics statement

This research did not include any human subjects and animal experiments.