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Antiproliferative Activity of *Pyracantha* and *Paullinia* Plant Extracts on Aggressive Breast and Hepatocellular Carcinoma Cells

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Abstract: In recent decades, the use of plants as a natural remedy has been widely applied in traditional medicine and the treatment of various diseases, including cancer. However, in order to confirm the potential benefits of anticancer drug development from natural sources, in-depth screening assessments are necessary. In the present study, we aimed to evaluate the cytotoxic effects of eight medicinal plants against breast carcinoma and hepatocellular carcinoma cell lines. Remarkably, among all the tested plant extracts, *Pyracantha angustifolia* and *Paullinia cupana* extracts showed maximum inhibition in the two cancer cell line models, as detected by cell viability assays, but not in normal mammary epithelial cells. Moreover, induction of cell cycle arrest was seen in both cancer cell models after treatment with extracts derived from the fruits of *P. angustifolia* and the seeds of *P. cupana*. Phytochemical and antioxidant analyses demonstrated the presence of high phenolic and flavonoid contents, including an increase in 2,2-diphenyl-1-picrylhydrazyl (DPPH) activity. The growth inhibition of human breast carcinoma and hepatocellular carcinoma cells mediated by both extracts appears to be associated with apoptosis and upregulated expression of pro-apoptotic genes (caspase-3, caspase-7, tumor suppressor protein-p53, cytochrome c, poly (ADP-ribose) polymerase, p53 upregulated modulator of apoptosis, and Bcl-2-associated X-protein). Together, these results indicate that *P. angustifolia* and *P. cupana* offer a promising approach for the development of anticancer agents. However, further detailed research is required to make these plants applicable for therapeutic use.

Keywords: hepatocellular carcinoma cells; breast carcinoma cells; flow cytometry; phytochemical analysis; plant extracts

1. Introduction

Despite the progress of modern therapeutic and diagnostic methods, cancer remains the most challenging disease for medical science. Breast cancer is the most frequently diagnosed cancer and the leading cause of mortality in women worldwide [1]. In addition, recent investigations on the incidence and mortality of liver cancer showed an estimated annual mortality rate of over 650,000 patients globally in 2017, particularly in Asian countries [2,3]. Currently, surgical interventions followed by chemotherapy and adjuvant radiation therapy are frequently used to improve the survival rate of cancer patients. On the other hand, natural products, including medicinal plants, are also routinely utilized in primary health care, mainly in developed countries. Since the emergence of ancient medicine, natural products and chemical compounds derived

from plants have been used to treat human diseases for centuries and have received increasing consideration for their potential as novel cancer therapeutic agents over the past several years [4]. An alternative approach to the use of traditional therapeutic methods involves the application of anticancer compounds derived from plants. Additionally, growing evidence suggests the possible use of plant-derived compounds to combat carcinogenesis and associated progression, underlining the significance of these products in cancer treatment. Interestingly, approximately 55% of anticancer drugs are currently formulated from natural products [5]. This expanding area of research continues to explore novel natural products from different plant sources, including the sea, which has a nearly endless supply of resources [6,7], facilitating the analysis of more selective, potent, and less toxic compounds to provide better therapeutic approaches for designing a wide range of new anticancer drugs. Here, we examined the effects of eight whole-plant extracts (methanol extraction) from different plant species on two human cancer cell lines: Breast carcinoma and hepatocellular carcinoma cell cultures. Among them, *Pyracantha angustifolia* (fruits) and *Paullinia cupana* (seeds) were then selected for additional detailed apoptosis research focusing on cell death markers elevated by these crude plant extracts. *Pyracantha* plants, which are found in South Korea, southern Europe, and southwest China, can reach up to 4.5 m. As an evergreen coniferous plant, *Pyracantha* has many branches and leaves, flowers, and fruits. Fico et al. showed that one *Pyracantha* species, *P. coccinea*, can be used in traditional medicine for diuretic, cardiac, and tonic purposes due to its phenolic and antioxidant activities [8]. In another study, *Pyracantha fortuneana (Maxim.)* exhibited satisfactory antitumor activity in breast and ovarian cancer cells due to the anti-mutation and anti-oxidation activities of its selenium-enriched polysaccharides [9]. *Paullinia cupana*, commonly known as guaraná, is a Brazilian plant that is frequently touted for its pharmacological activities in neurodegenerative disorders, such as Parkinson's disease, due to the high content of methylxanthines and tannins in its seeds [10]. Oliveira et al. suggested that the administration of *P. cupana Mart. var. Sorbilis* defends neuroblastoma cells from rotenone-induced cytotoxicity [11].

In this study, we hypothesized that different plant extracts might contain diverse molecules with antitumor activities and are very effective in selective cancer cell death. After screening eight medicinal plant extracts, we investigated the anticancer effects of *P. angustifolia* and *P. cupana* extracts in more detail as they showed maximum inhibitory activity on breast carcinoma and hepatocellular carcinoma cells without affecting their normal counterparts. We observed that *Pyracantha* and *Paullinia* exerted a protective effect in both cancer cell line models, as observed by cell proliferation assays. Analysis with propidium iodide (PI)/RNase revealed that the treatment extracts induced cell cycle arrest with increased expression levels of proteins associated with apoptosis (programmed cell death) in cancer cell line models. Taken together, our results suggest that these two plant extracts demonstrate strong anticancer capabilities by inhibiting cancer cell growth.

2. Experimental Section

2.1. Collection and Identification of Plant Materials

All the plants used in this study were collected, identified, and kindly provided by the Southern Plant Resources Institute (Jeju, Korea). The plant properties are summarized in Table 1.

Table 1. Overview of eight plant extracts, including their medicinal uses and growing location details.

Plant	Family	Medical Uses	Location	Part Used
Pyracantha angustifolia	Rosaceae	Used as a cure for blood circulation, diarrhea, dysentery, and anti-ulcer activity [12]	China	Fruits
Saururus chinensis	Saururaceae	Eduma, gonorrhea, and jaundice [13]	Jeju-do (Korea)	Whole plant
Sorbus commixta	Rosaceae	Protecting the stomach and intestines and pulmonary tuberculosis [14,15]	Japan, Korea Ulleung-do	Branches, shells, and berries of dried margarine
Delonix regia	Fabaceae	Anti-microbial [16], Anti-diabetic agent [17]	Africa	Leaves
Paullinia cupana	Asteraceae	Cognitive ability [18], hepatoprotective [19]	Brazilian amazon basin	Seeds
Ficus erecta	Moraceae	Neuralgia [20], Rheumatoid arthritis [21]	South Korea, Japan	Leaves
Quercus salicina	Fagaceae	Vasorelaxant [22], urinary stones [23], and dermatitis [24]	Japan, South Korea, Taiwan	Whole plant
Colocasia esculenta	Araceae	Antimicrobial [25], antioxidant [26], and anticancer [27]	Asia (Indian subcontinent)	Leaves

2.2. Extract Preparation

Prior to extraction, the desired plant parts were powdered using a blender, placed in 70% ethanol, maintained in a shaking incubator for 2 days, and then filtered through filter paper. The filtered extract was concentrated under reduced pressure at 70 °C using a rotary pressure concentrator and powdered using a freeze dryer. The freeze-dried concentrated plant extracts were stored at –20 °C until use. Treatments were performed with different dilutions from the obtained extracts and used for further cell experiments to measure anticancer activities (Figure 1).

2.3. Cell Culture and Extract Treatments

Human breast carcinoma MDA-MB231 and hepatocellular carcinoma HEPG2 cells were purchased from the Korean Cell Line Bank (Seoul, Korea). The normal human mammary epithelial cell line MCF10A was kindly gifted by Seoul National University (Seoul, Korea). MDA-MB231 and HEPG2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U·mL^{-1} of penicillin, and 100 µg·mL^{-1} of streptomycin (Gibco, Waltham, MA, USA). MCF10A cells were cultured using a Mammary Epithelial Cell Growth Medium BulletKit^{TM} (Lonza, Basel, Switzerland) according to the manufacturer's instructions. All cells were maintained in a humidified incubator at 37 °C with 5% CO_2 and passaged every 2 to 3 days. After reaching the desired confluence, the cells were seeded for the respective experiments and treated with various concentrations (10, 40, 160, or 320 µg·mL^{-1}) of plant extracts. Stock concentrations were prepared in dimethyl sulfoxide (DMSO), and hydrogen peroxide was purchased from Sigma-Aldrich (Seoul, Korea).

2.4. Radical Scavenging Activity [1,1-diphenyl-2-picrylhydrazyl (DPPH) Test]

The effects of the plant extracts on DPPH [28] radicals were estimated according to the procedure in the literature [29]. To this end, the sample solution (1 mL) was added to 4 mL of a 0.004% methanol solution of DPPH. Ascorbic acid (Sigma-Aldrich) was used as the standard to measure DPPH activity in the samples. The colored test sample absorbance was read at 517 nm in the dark at room temperature

after 25 min of incubation using an enzyme-linked immunosorbent assay reader (Epoch; BioTek Instruments, Inc., Winooski, VT, USA).

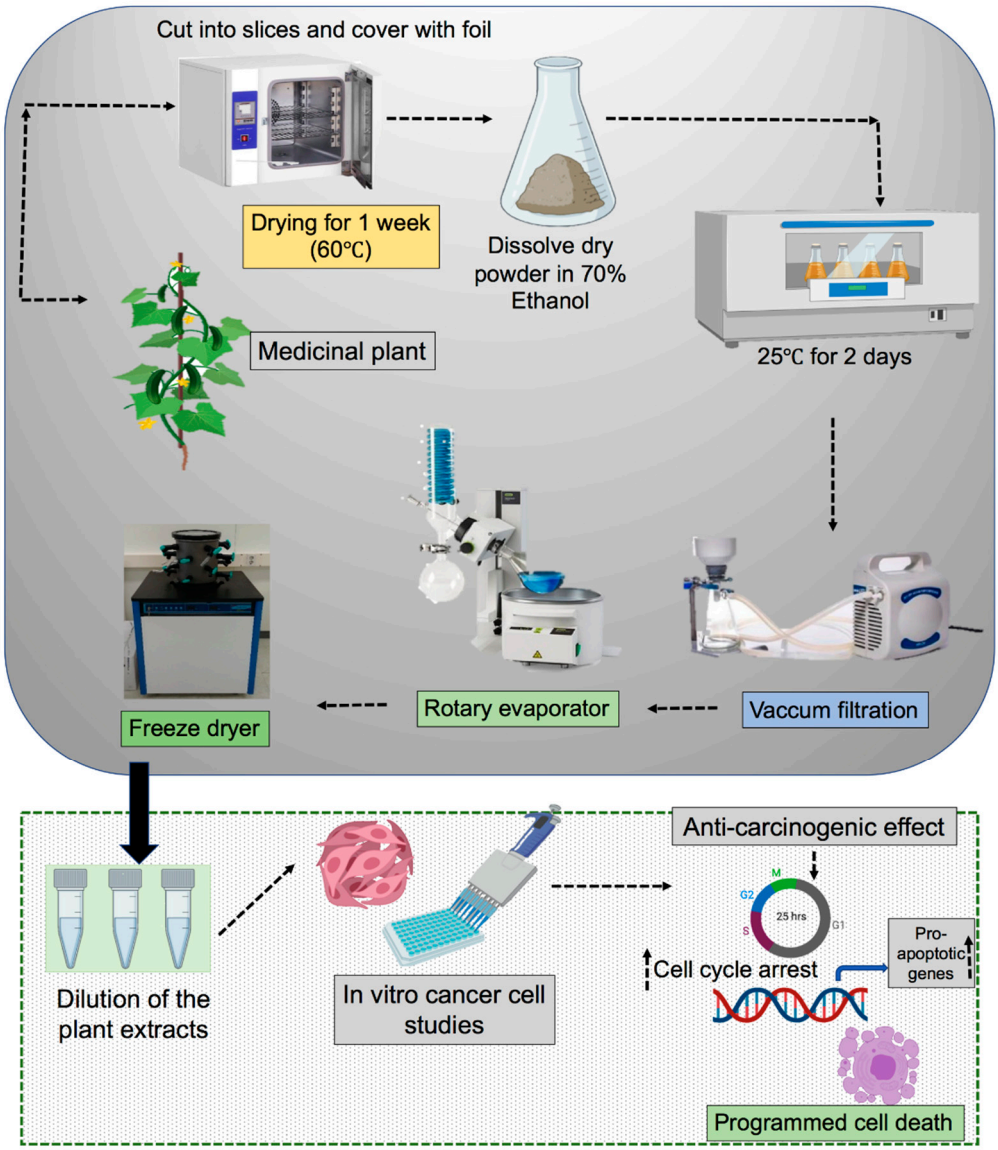

Figure 1. Schematic illustrations of the extract preparation using various medicinal plants and the cancer cell experimental protocol.

2.5. Determination of Total Bioactive Components

Total phenolic and flavonoid contents were determined by employing the methods previously described in the literature [30]. To measure the phenolic contents, gallic acid (0.04–200 μg·mL^{-1}) was used as the standard. The respective concentrations of phenolic compounds present in *P. angustifolia* and *P. cupana* were expressed in mg of gallic acid equivalents/g of each extract. However, in the case of flavonoids, quercetin (0.04–200 μg·mL^{-1}) was used as the standard, and the total flavonoid contents were expressed in mg of quercetin equivalents (QE)/g of each extract.

2.6. Cell Viability Assay

After 24 h of cell seeding in a 96-well plate, the cancer and normal cells were treated with different extract concentrations (10, 40, 160, or 320 μg·mL^{-1}), and cell viability was assessed using alamarBlue (AB; Thermo Fisher Scientific, Waltham, MA, USA) dye after 24, 48, and 72 h of incubation.

Briefly, an AB solution (10% v/v) was prepared in the medium, added to each well, further incubated for 1–2 h, and measured using a plate-reading spectrometer (BioTek Instruments, Inc.), as described in our earlier work [31]. Monitored fluorescence was considered a measure of AB dye conversion in the treated and untreated samples. IC50 values were calculated using GraphPad Prism software, where the Fit Spline smoothing curve fitting function was used.

2.7. Cell Cycle Arrest

Seventy-two hours post-treatment with the extracts, the cells were harvested and washed with ice-cold phosphate-buffered saline (PBS) followed by fixation, and permeabilized with 70% EtOH at 4 °C for 12 h. Later, the cells were further washed, resuspended in a staining solution comprising 1 $\mu g \cdot mL^{-1}$ RNase A with 5 $\mu g \cdot mL^{-1}$ PI, and incubated in the dark for an additional 20–25 min. After incubation, the stained cells were directly analyzed using a flow cytometer (BD FACSVerse; BD Biosciences, Franklin Lakes, NJ, USA) and FACSuite software. In each test group, 10,000 events/sample were detected.

2.8. Intracellular Reactive Oxygen Species (ROS) Detection

For intracellular detection, the cells were stained with 2′,7-dichlorodihydroflourescein diacetate for 30 min. The cells were washed with cold PBS and analyzed immediately using the BD FACSVerse cytometer and FACS suite software (BD Biosciences) [32].

2.9. Caspase Activity Detection

Caspase-3/7 and caspase-9 activities were measured after 72 h of extract treatment, and all procedures were performed using Caspase-Glo®3/7 and Caspase-Glo®9 Assay kits, respectively (Promega, Seoul, Korea). Luminescence was recorded using a microplate reader in all untreated and treated samples of both MDA-MB231 and HEPG2 cancer cells.

2.10. Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) Analysis

TRIzol reagent (RNAiso Plus; Takara Bio, Shiga, Japan) was used to isolate RNA from the cells following the manufacturer's instructions. Total RNA (2 μg) was used to synthesize the template complementary DNA using MMLV Reverse Transcriptase (Enzymonics, Daejeon, Korea) SuperMix containing 30 mM $MgCl_2$, 750 mM KCl, RT buffer [500 mM Tris-HCl (pH 8.3) and 100 mM DTT], RNase inhibitor, and dNTPs as per the manufacturer's instructions. The mRNA expression levels of apoptosis-related genes were analyzed with qRT-PCR on an iCycler IQ Real-Time Detection System (Bio-Rad, Hercules, CA, USA).

2.11. Fourier-Transform Infrared (FTIR) and Ultraviolet-Visible (UV-Vis) Spectroscopy Analysis

To examine the presence of active constituents in each extract, FTIR and UV-Vis spectroscopy were used. All eight extracts were placed in the infrared beam output of the FTIR using a Shimadzu QATR-S spectrometer (Kyoto, Japan). Analysis of the acquired oscillatory spectra allows for the estimation of the functional groups with which the radiation interacted. The UV-Vis spectrum of each extract was recorded from diluted extracts (1 $mg \cdot mL^{-1}$). Each mixture was scanned using a circular dichroism spectrophotometer (JASCO J-815, JASCO, Inc., Easton, MD, USA).

2.12. Statistical Analysis

All data are expressed as the means ± standard deviations of triplicate measurements from three independent experiments. Significant differences between groups were analyzed using the Student's *t*-test. Multiple group comparisons were made using one-way analysis of variance. Levels of significance are indicated by $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. Nonsignificant groups are denoted as ns.

3. Results

3.1. Analysis of Active Constituents in the Eight Plant Extracts Using FTIR and UV-Vis Spectroscopy

Plant extracts are mixtures of multiple complex phytochemicals, such as organic acids, polyphenols, and polysaccharides [33,34]. To identify the existing functional groups of the plant extracts, we performed chemical analysis techniques, including FTIR and UV-Vis spectroscopy. Figure 2A,B show the measured FTIR spectra of the plant extracts. All of the plant extract spectra show the presence of OH stretching (3308-3318), C-H stretching (2830-2944), O=C=O stretching (2339-2365), C=O stretching (2028-2038), N-H bending (1585-1609), C-H bending (1401-1452), C-O stretching (1108-1111), and C-N stretching (1018-1022). The UV-Vis spectra of the eight plant extracts have absorption bands in the range of 260–400 nm, which indicate the absorption of polyphenols (Figure 2C,D) [35].

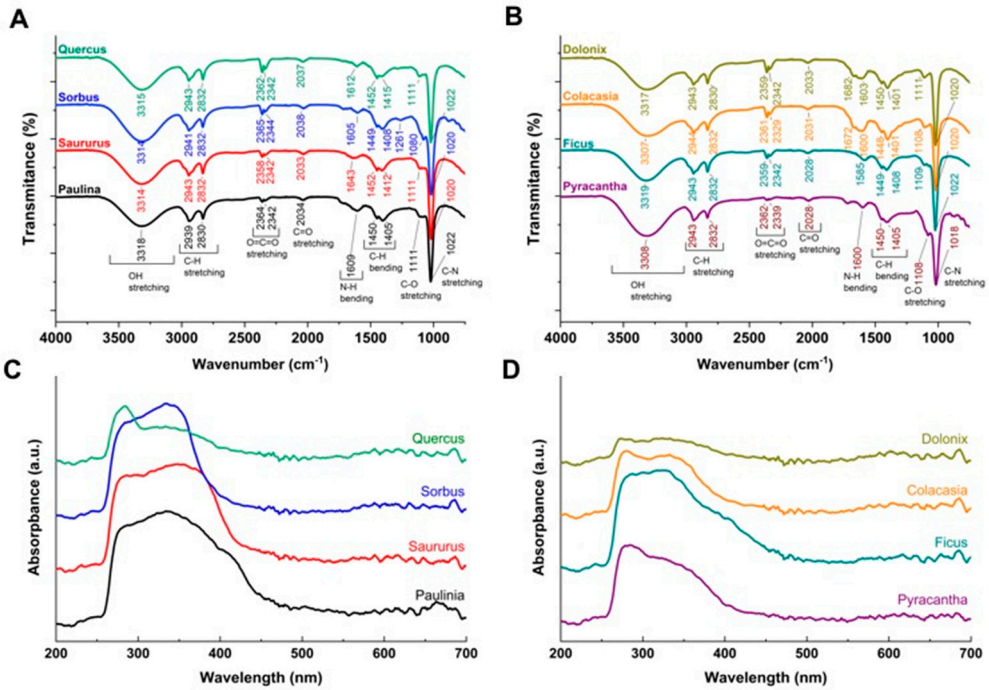

Figure 2. Analysis of phytochemical constituents in all eight plant extracts, including *Pyracantha angustifolia* and *Paullinia cupana*. Fourier-transform infrared (FTIR) spectra of (**A**) *Quercus, Sorbus, Saururus*, and *Paulina*; and (**B**) *Dolonix, Colacasia, Ficus*, and *Pyracantha*. UV-Vis absorption spectra of (**C**) *Quercus, Sorbus, Saururus*, and *Paulina*; and (**D**) *Dolonix, Colacasia, Ficus*, and *Pyracantha*.

3.2. Antiproliferative Activity Screening of the Eight Medicinal Plants

In a prescreening of the eight medicinal plants, we tested various concentrations of the plant extracts ranging from 10 to 320 μg·mL^{-1} against two cancer cell lines: Breast carcinoma MDA-MB231 and hepatocellular carcinoma HEPG2. For plant extract preparation, the parts used are mentioned in Table 1. After attachment, the cancer cell lines were treated with 0, 10, 40, 160, or 320 μg·mL^{-1} of plant extracts and maintained for the next 72 h to determine time-dependent effects. The results indicate that increasing concentrations of all plant extracts showed cell viability reduction in a concentration-dependent manner at all time points. Remarkably, most of these extracts were able to decrease cell viability by over 50% in both MDA-MBA231 and HEPG2 cancer cells as detected by alamarBlue assays. However, *Pyracantha* and *Paullinia* showed the maximum growth inhibition in both types of cancer cell models. The percentage of cell viability in comparison to the vehicle-treated control cells is shown in Figures 3 and 4. We also calculated the half maximal inhibitory concentration (IC_{50}) values of each plant extract against both MDA-MB231 and HEPG2 cancer cells. In the case of MDA-MB231 cells, the IC_{50} values were as follows: *Paullinia* (43.1±10.69 μg·mL^{-1}), *Saururus* (47.46±5.78 μg·mL^{-1}),

Sorbus (45.5±22.90 μg·mL^{-1}), *Quercus* (56.00±15.61 μg·mL^{-1}), *Pyracantha* (21.91±2.10 μg·mL^{-1}), *Ficus* (47.93±17.92 μg·mL^{-1}), *Colocasia* (49.3±11.53 μg·mL^{-1}), and *Delonix* (76.08±18.96 μg·mL^{-1}). For HEPG2 cells, the IC_{50} values were as follows: *Paullinia* (32.57±7.60 μg·mL^{-1}), *Saururus* (42.50±7.58 μg·mL^{-1}), *Sorbus* (33.50±5.42 μg·mL^{-1}), *Quercus* (34.44±14.95 μg·mL^{-1}), *Pyracantha* (28.41±4.53 μg·mL^{-1}), *Ficus* (43.59±13.2 μg·mL^{-1}), *Colocasia* (39.46±7.29 μg·mL^{-1}), and *Delonix* (34.59±3.45 μg·mL^{-1}). The IC_{50} values confirm that *Pyracantha* and *Paullinia* showed the lowest IC_{50} against both cancer cell types among all extracts tested (Supplementary Table S1). Since, the reduction in viability was not consistent with increasing concentration of plant extract. Therefore, our IC_{50} values were higher, calculated from the Prism-based fit Spline, the smoothing curve fitting function, as expected from viability data results. Furthermore, when both these extracts were tested on normal MCF10A mammary cells, *Pyracantha* and *Paullinia* did not display any cell toxicity (Figure 5). These results suggest that *Pyracantha* and *Paullinia* may have high potential for the inhibition of cell growth against different cancer cell types without inducing much toxicity.

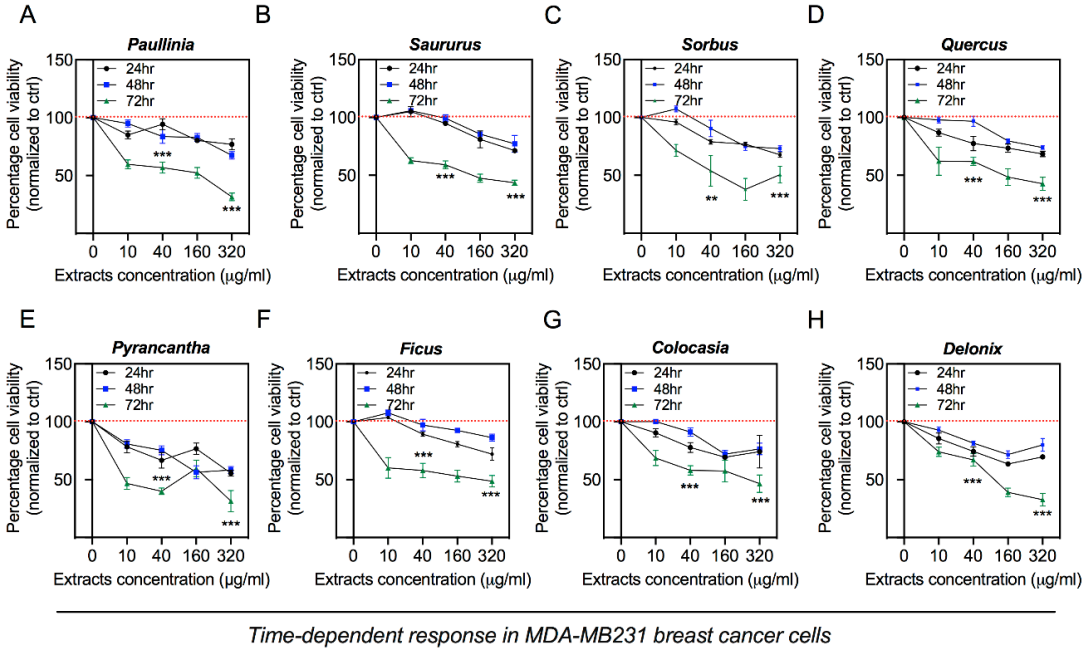

Figure 3. Cell viability screening analysis of different extracts from eight medicinal plants in human breast carcinoma MDA-MB231 cells. The alamarBlue assay was performed in *Paullinia* (**A**), *Saururus* (**B**), *Sorbus* (**C**), *Quercus* (**D**), *Pyracantha* (**E**), *Ficus* (**F**), *Colocasia* (**G**), and *Delonix* (**H**) extract-treated (0, 10, 40, 160, or 320 μg·mL^{-1}) cells after 24, 48, and 72 h of incubation. $^{**}p < 0.01$; $^{***}p < 0.001$. Untreated samples were used as controls for all tested plant extracts.

3.3. Pyracantha and Paullinia Induce Cell Cycle Arrest

Next, to investigate whether the inhibitory effects of these two extracts were associated with cell cycle alteration, we performed cell cycle analysis in MDA-MB231 and HEPG2 cancer cells using flow cytometry. In both cancer cell types, after treatment with plant extracts for 72 h, cell cycle distribution was analyzed using PI staining. Our results showed that treatment with both 40 and 320 μg·mL^{-1} concentrations of *Pyracantha* and *Paullinia* decreased the number of cells in the G0/G1 phase in MDA-MB321 and HEPG2 cancer cells (Figure 6A,B; Supplementary Table S2). An accumulation of cells was also observed in the G2/M phase using these concentrations of both extract samples, which was more significant in HEPG2 cells (Figure 6C,D). However, differences can be observed in the Sub-G1 phase between control samples and treatments for the MDA-MB-231 cells. Our data suggest that *Pyracantha* and *Paullinia* extracts are capable of inducing DNA cell cycle arrest in cancer cells.

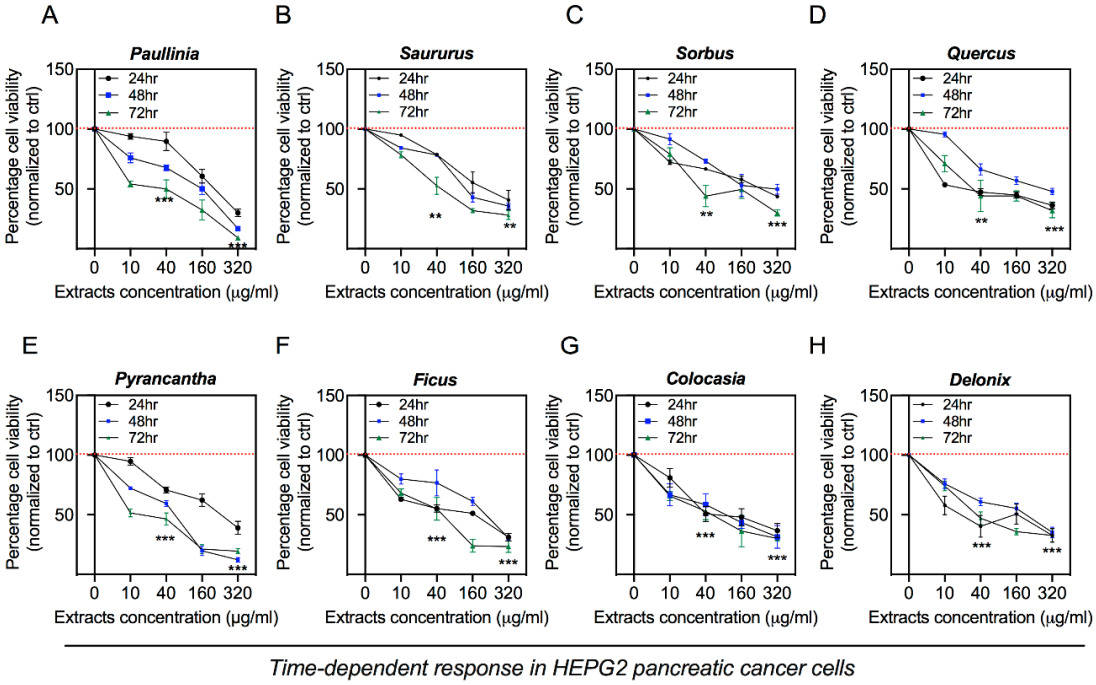

Figure 4. Cell viability screening analysis of different extracts from eight medicinal plants in human hepatocellular carcinoma HEPG2 cells. The alamarBlue assay was performed in *Paullinia* (**A**), *Saururus* (**B**), *Sorbus* (**C**), *Quercus* (**D**), *Pyracantha* (**E**), *Ficus* (**F**), *Colocasia* (**G**), and *Delonix* (H) extract-treated (0, 10, 40, 160, or 320 μg·mL^{-1}) cells after 24, 48, and 72 h of incubation. $^{**}p < 0.01$; $^{***}p < 0.001$. Untreated samples were used as controls for all tested plant extracts.

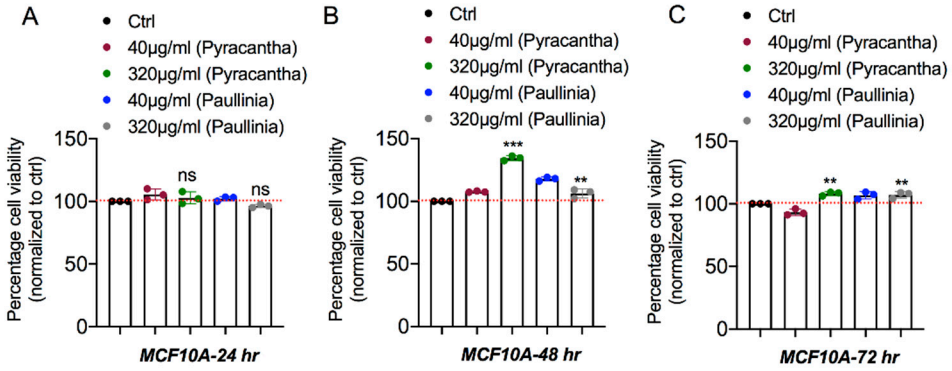

Figure 5. *Pyracantha* and *Paullinia* did not affect normal cell viability. (**A**–**C**) alamarBlue assays in normal MCF10A mammary epithelial cells treated with two different concentrations (40 or 320 μg·mL^{-1}) of *Pyracantha* and *Paullinia* after 24, 48, and 72 h of incubation. $^{**}p < 0.01$; $^{***}p < 0.001$. Nonsignificance is denoted as ns. Untreated samples were used as controls for all tested plant extracts.

3.4. *Pyracantha and Paullinia Stimulate Apoptotic Cell Death*

The ability of *Pyracantha* and *Paullinia* to suppress cell growth and stimulate cell cycle arrest in cancer cells motivated us to examine whether these extracts have potential to induce programmed cell death or apoptosis. It is well known that apoptotic cell death is carried out by caspases (cysteine aspartyl-specific proteases), which are cysteine proteins that cleave target proteins [36–38]. With this goal in mind, we measured caspase-3/7 and caspase-9 activities in both MDA-MB231 and HEPG2 cancer cells treated with 40 or 320 μg·mL^{-1} extract concentrations. The data showed that *Pyracantha* and *Paullinia* increased the caspase activities in both cell types (Figure 7A–D). Remarkably, this effect was more prominent in *Paullinia*-treated cells upon comparison of both cell types. To verify these phenomena, we assessed the mRNA expression levels of apoptosis-related genes. Treatment

with the 320 μg·mL^{-1} concentration of *Pyracantha* and *Paullinia* extracts significantly upregulated the expression levels of caspase-3 (*Casp3*), caspase-7 (*Casp7*), tumor suppressor protein-p53 (*p53*), poly (ADP-ribose) polymerase (*Parp*), and p53 upregulated modulator of apoptosis (*Puma*) in HEG2 cancer cells (Figure 8). Interestingly, high expression of cytochrome c (*Cytc*) suggested the possibility of a mitochondria-mediated apoptosis pathway. These findings indicate that *Pyracantha* and *Paullinia* decrease cancer cell growth through apoptosis induction.

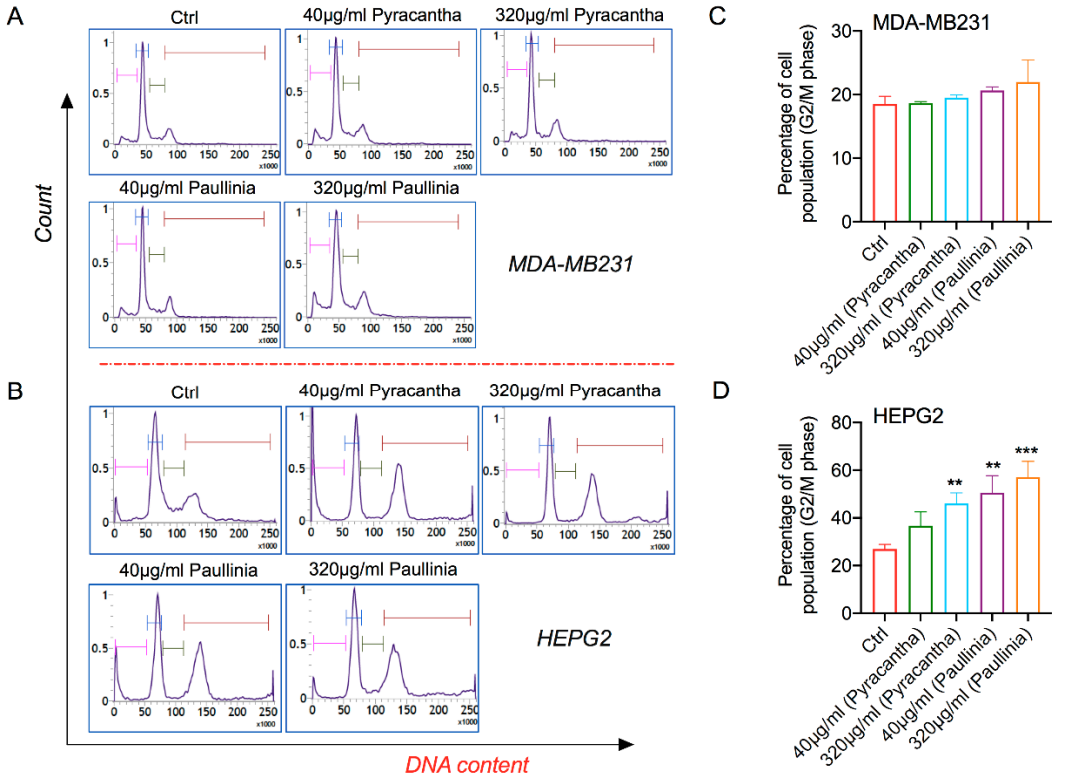

Figure 6. *Pyracantha* and *Paullinia* stimulate cell cycle arrest in both cancer cell types. (**A** and **B**) PI/RNase A-stained cells treated with two different concentrations (40 or 320 μg·mL^{-1}) of *Pyracantha* and *Paullinia* were analyzed using flow cytometry. The cell populations are marked as sub-G1 (pink), G0/G1 (blue), S (green), and G2/M (brown) in histograms. (**C** and **D**) The representative graph shows the percentage of the cell population in the G2/M phase in MDA-MB231 and HEPG2 cancer cells, respectively. Untreated samples were used as controls for all tested plant extracts.

3.5. Detection of Polyphenols in P. angustifolia and P. cupana Extracts and Intracellular Redox Status

In plants, antioxidant activity is mainly due to the presence of phenolic compounds, including polyphenols and flavonoids. Flavonoids are also considered critical antioxidants in herbal medicine [39,40]. Our data showed that *P. angustifolia* and *P. cupana* have high levels of phenolic, as well as flavonoid, contents (Figure 9A,B). Our study results confirmed that the fruits and seeds of *P. angustifolia* and *P. cupana* are good sources of phenols and flavonoids (polyphenols). Therefore, we postulated that both these extracts could have antioxidant potential. To test this possibility, we performed the DPPH scavenging assay, the reaction of which is based on the changing color of free radical solution with tested substances. The decline in absorption values after adding extracts to the radical solution is directly proportional to the quantity of produced DPPH [41]. DPPH measurement was conducted with two different concentrations of extracts, 40 and 320 μg·mL^{-1}. Remarkably, the highest capacity for DPPH inhibition was observed using the 320 μg·mL^{-1} concentration with 73% and 48% in *Pyracantha* and *Paullinia* extracts, respectively (Figure 9C). These findings suggest the presence of phenolic and flavonoid compounds in both extracts. To further investigate whether the extracts of *Pyracantha* and *Paullinia* inhibit the growth of cancer cells by reducing ROS generation, we monitored

the redox status of the HEPG2 extract-treated cells using the oxidation-sensitive fluorescent dye DCFDA. Both extracts showed the smallest effect on ROS generation at the lower concentration (40 μg·mL^{-1}). Thus, *Pyracantha* and *Paullinia* extracts decreased ROS generation in HEPG2 cells in a concentration-dependent manner (Figure 9D,E). ROS generation decreased more significantly at the higher concentration (320 μg·mL^{-1}), which may be due to increased apoptotic cell death. Earlier reports have suggested that various natural compounds present in plants are capable of combating cancer via inhibition of ROS generation in patients [42,43].

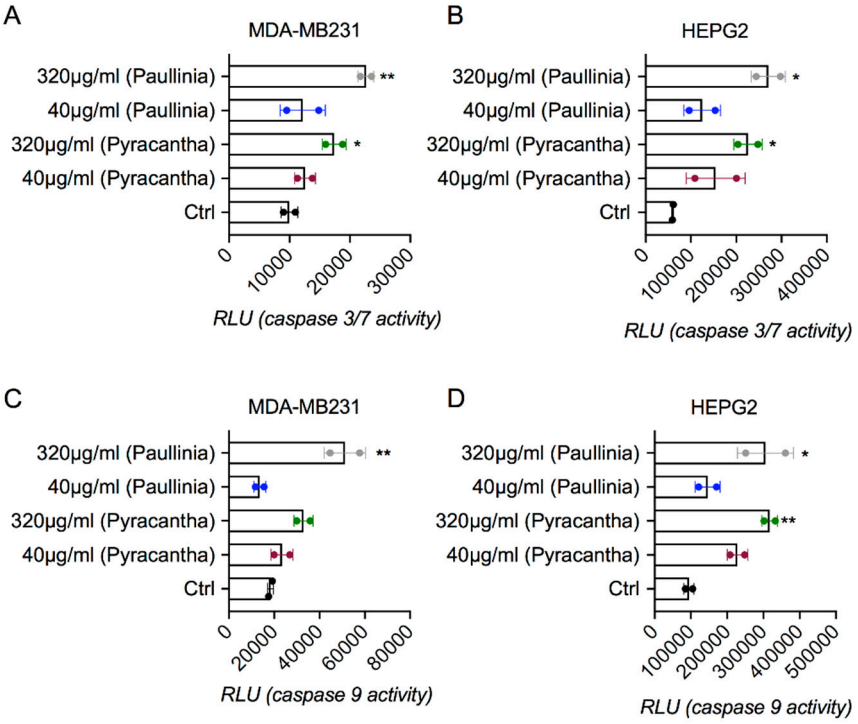

Figure 7. *Pyracantha* and *Paullinia* induce apoptosis in cancer cells. (**A** and **B**) Caspase-3/7 activity was measured in MDA-MB231 and HEPG2 cells treated with two different concentrations (40 or 320 μg.ml^{-1}) of *Pyracantha* and *Paullinia* extracts, respectively. (**C** and **D**) Caspase-9 activity was measured in MDA-MB231 and HEPG2 cells treated with two different concentrations (40 or 320 μg.ml^{-1}) of *Pyracantha* and *Paullinia* extracts, respectively.*$p < 0.05$; **$p < 0.01$. Untreated samples were used as controls for all tested plant extracts.

Based on this finding, we further assessed the expression levels of antioxidant enzymes, such as catalase (CAT) and manganese-dependent superoxide dismutase (MnSOD or SOD2), in the extract-treated HEPG2 cells. The CAT and SOD2 levels were upregulated at the higher concentration (320 μg·mL^{-1}) in HEPG2 cancer cells treated with both plant extracts (Figure 9F). These results suggest that antioxidants present in *Pyracantha* and *Paullinia* induce the inhibition of cancer cell proliferation through decreased ROS levels. Additionally, we have performed a cell viability test in HEPG2 cells in the presence of H_2O_2 with both Pyracantha and Paullinia extracts to further validate our findings. Interestingly, H_2O_2 blocked the effect of both plant extracts on the decrease in viability in cancer cells at the 50 μM concentration (Supplementary Figure S1).

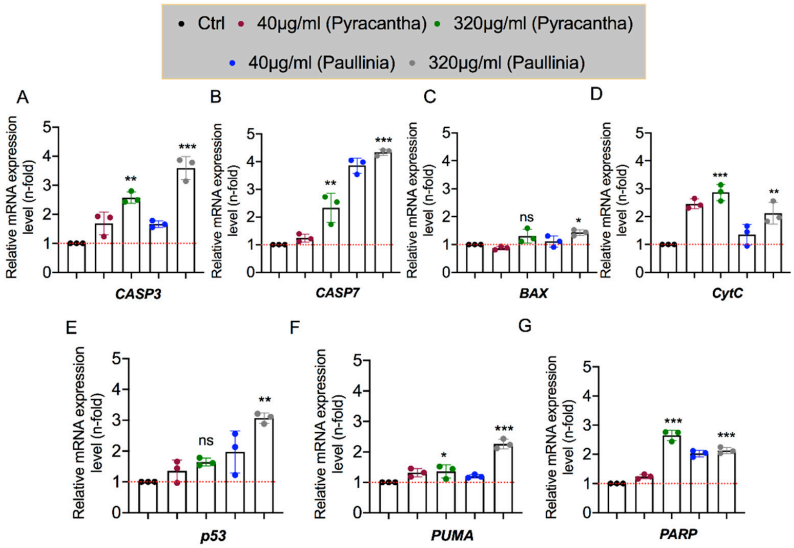

Figure 8. Apoptosis-related genes were upregulated by *Pyracantha* and *Paullinia* in cancer cells. (**A**–**G**) Detection of the mRNA expression levels of *Casp3*, *Casp7*, *BAX*, *Cytc*, *p53*, *Puma*, and *Parp* in HEPG2 cells treated with two different concentrations (40 or 320 µg·mL^{-1}) of *Pyracantha* and *Paullinia* after 72 h. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. Non-significance is denoted as ns. Untreated samples were used as controls for all tested plant extracts.

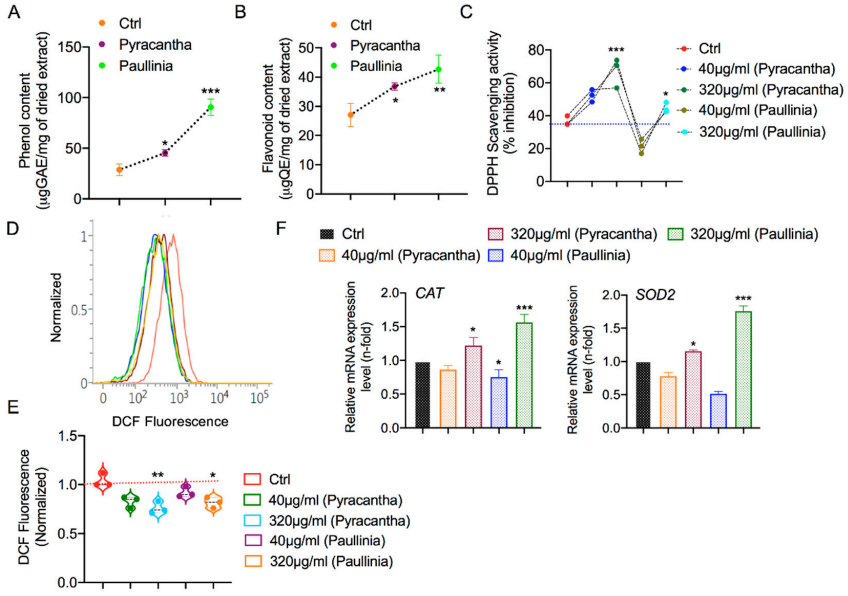

Figure 9. Determination of phytochemical components and antioxidant activity in *Pyracantha* and *Paullinia* extracts. Analysis of phenolic compounds (**A**), flavonoids (**B**), and antioxidant scavenging activity (**C**) in *Pyracantha* and *Paullinia* extracts. (**D** and **E**) Intracellular reactive oxygen species (ROS) levels in HEPG2 cancer cells following treatment with *Pyracantha* and *Paullinia* extracts at the indicated concentrations. (**F**) qPCR analysis of *CAT* and *SOD2* (*MnSOD)* antioxidant levels in HEPG2 cancer cells following treatment with *Pyracantha* and *Paullinia* extracts at the indicated concentrations. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

4. Discussion

The extraction of bioactive compounds, such as phenols, from natural products or plant material is influenced by their method of extraction, chemical structure, and the time and conditions of storage [44]. A growing number of studies show that most medicinal plants exhibit antioxidant and anticancer properties due to the presence of chemical components. Antioxidants protect against infections

and disease in humans by neutralizing the effects of free radicals. In particular, phenols possess a structural component for free radical scavenging and thus have antioxidant potential [45,46]; as a result, phenols have attracted considerable interest in the field of medicine. Many research studies have proven the antioxidant and anticancer potential of several natural plant extracts in a wide variety of human systems and animal models with relevance to disease [47]. Extracted phenolic compounds show strong protective effects via anti-inflammatory, anti-mutagenic, and anticancer activities [48]. Free radicals play a key role in a wide range of cellular processes by damaging proteins and DNA, leading to many fatal diseases, including cancer [49,50]. Antioxidants can be used for effectively inhibiting cellular proliferation, promoting cell apoptosis, inhibiting gene expression, enhancing detoxification enzymes, and scavenging ROS. Thus, by suppressing oxidative stress and free radicals, phenols and flavonoids promote the amelioration of DNA damage, decreasing the amount of abnormal cell division [51]. In this way, many antioxidant-rich plants exhibit anticancer potential [52,53].

Antioxidants, particularly polyphenols, have been demonstrated as promising agents that can delay cancer initiation and progression [54]. It is believed that flavones may exert their anticancer effects via several possible mechanisms, such as the alteration of cancer cell signaling pathways, removal of carcinogenic agents, antioxidant enzymatic actions, cell cycle arrest, and induction of apoptosis [55]. Most of the anticancer properties correlated with flavanols are supposed to be facilitated by the flavanol epigallocatechin gallate, which has been revealed to prevent cancer cell growth by modifying the expression of cyclin proteins involved in the cell cycle, as well as the activity of cellular signaling proteins involved in proliferation and transformation [56]. In our study, most of the tested medicinal plant extracts protected against breast carcinoma and hepatocellular carcinoma-induced cytotoxicity at a higher concentration (320 $\mu g \cdot mL^{-1}$). However, *Pyracantha* and *Paullinia* showed the maximum level of inhibition against two different cancer cell types (Figures 2 and 3). It is worth mentioning that both these extracts had no toxic effects on the normal counterparts of the cancer cells, as confirmed in mammary epithelial MCF10A cells (Figure 4). *Pyracantha* and *Paullinia* induced cell cycle arrest to stimulate apoptosis in cancer cells, which was consistent in both cancer cell types. A recent report also suggested that another species, *P. fortuneana*, inhibits cancer cell growth and induces apoptosis due to the presence of selenium-rich polysaccharides [9,57]. Apoptotic cell death in cancer cells may be the means of an intrinsic mitochondria-mediated pathway [58]. In this study, we examined the expression of p53, which is known as a cell cycle regulator that induces cell cycle arrest via Puma transcription, leading to apoptosis in response to many cellular stressors [59,60]. Moreover, p53 translocates to the mitochondria for subsequent release of cytochrome c and pro-caspase-3 activation according to nontranscriptional mechanisms [61]. In addition, p53 physically interacts with the anti-apoptotic protein Bcl-2 through its DNA-binding domain, thus leading to sequestration of pro-apoptotic BAX/BAK proteins and causing the release of mitochondrial cytochrome c in the cells to induce apoptosis [62]. In agreement with these studies, we detected an increase in p53 in both extract-treated cancer cell types, which may induce apoptotic death by similar mechanisms. Notably, *Pyracantha* and *Paullinia* appear to upregulate the expression of other apoptosis-related genes, such as *Casp3, Parp, Puma, Cytc, Casp7,* and Bcl-2-associated X-protein. Accordingly, further studies are required to fully understand the precise mechanism of *Pyracantha* and *Paullinia*-induced apoptosis.

Here, we show that high phenol and flavonoid contents are present in the seed and fruit extracts of *Pyracantha* and *Paullinia*, which result in significant cancer inhibition activity with strong antioxidant activity. Our results demonstrate that both extracts have potent antioxidant capacities for DPPH radicals, which were highly consistent with their phytochemical profiles. It can be assumed from the results presented here that remarkable sensitivity could be achieved in different cancer types by using both these extracts, which suggests their potential as therapeutic candidates for novel anticancer approaches.

5. Conclusions

In the current study, we found that *Pyracantha* and *Paullinia* extracts exhibit significant antioxidant activity due to high phenolic and flavonoid contents. Additional studies on the underlying mechanism of action of these extract compounds are ongoing. Based on the present findings using in vitro experiments, *Pyracantha* and *Paullinia* appear to be strong natural antioxidants that may have an impact on the treatment of various cancers, as well as other human diseases.

Supplementary Materials: The following are available online at http://www.mdpi.com/2076-3417/10/21/7543/s1, Table S1: Cytotoxicity (IC50 values µg/ml) for eight plant extracts against MDA-MB231 and HEPG2 cancer cell line calculated at 72 hr (n = 3) using GraphPad prism software based on fit spline smoothing curve fitting function., Table S2: Effects of Pyracantha and Paullinia plant extracts on the cell cycle of cancer cell lines MDA-MB231 and HEPG2. Percentage of cancer cells in the sub-G1, G0/G1, S and G2/M phase were calculated using FACS suite software., Figure S1: Effects of Pyracantha and Paullinia plant extracts on the cell viability of HEPG2 cancer cells in presence of H_2O_2 treatment (50µM concentration). * $p < 0.05$.

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