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Original Research

A Standardized Extract of *Petasites hybridus* L., Containing the Active Ingredients Petasins, Acts as a Pro-Oxidant and Triggers Apoptosis through Elevating of NF-κB in a Highly Invasive Human Breast Cancer Cell Line

Sonia Apostolova[1,*], Tsvetelina Oreshkova[2], Veselina Uzunova[1], Irina Georgieva[1], Liliana Maslenkova[1], Rumiana Tzoneva[1,*]

[1]Laboratory of Transmembrane Signaling, Institute of Biophysics and Biomedical Engineering, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria
[2]Institute of Biology and Immunology of Reproduction "Acad. Kiril Bratanov", Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria
*Correspondence: sonia_apostolova@yahoo.com (Sonia Apostolova); tzoneva@bio21.bas.bg (Rumiana Tzoneva)
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Abstract

Background: Common butterbur (*Petasites hybridus* L.) is a traditional medicinal plant with numerous therapeutic properties among which is its recently uncovered anti-tumor activity. The present study aims to examine the activity of a standardized Bulgarian *Petasites hybridus* L. root extract, containing the active ingredients petasins, on the human breast cancer cell line MDA-MB-231 and non-cancerous MCF-10A cells. Specifically, we examined cell death, oxidative stress, and nuclear factor kappa-B (NF-κB) signaling. **Methods**: A standardized butterbur powdered extract containing a minimum of 15% petasins was used. A lipophilic extract was obtained from subterranean portion of the plant of Bulgarian populations of *Petasites hybridus* using liquid-liquid extraction after completely removing pyrrolizidine alkaloids. The induction of apoptosis and necrosis was analyzed by flow cytometry, and oxidative stress biomarkers and NF-κB were measured using enzyme-linked immunosorbent assay (ELISA). **Results**: *Petasites hybridus* L. root extract triggered apoptosis in a cancer-specific fashion and induced a moderate oxidative stress characterized by diminished glutathione (GSH) levels and elevated malondialdehyde (MDA) levels in MDA-MB-231 72 h after treatment. NF-κB levels were higher in cancer cells after treatment with IC_{50} and IC_{75} doses, this suggested that the NF-κB pathway was activated in response to oxidative stress leading to the induction of apoptosis. MCF-10A cells were affected to a lesser extent by the *Petasites hybridus* extract, and the adaptive response of their antioxidant defense system halted oxidative stress. **Conclusions**: Overall, these results indicate that *Petasites hybridus* L. root extract selectively acts as a pro-oxidant in breast cancer cells and thus represents a potential therapeutic option for cancer treatment with fewer side effects.

Keywords: *Petasites hybridus* L.; petasins; apoptosis; oxidative stress; NF-κB; anticancer effects

1. Introduction

Despite current progress in cancer treatment, breast cancer death rates continue to increase each year in women worldwide [1]. The severe toxicity and multidrug resistance (MDR) of conventional chemotherapeutics remain significant challenges in the treatment of this disease. Hence, efforts need to focus on finding effective treatment strategies with minimized side effects on healthy cells and tissues. A number of investigations regarding the use of natural products and herbal extracts as anti-tumor therapeutics have increased in the last years due to favorable safety profiles and the possibility for long-term patient treatment.

The common butterbur (*Petasites hybridus* L.) is a medicinal plant of the Asteraceae family and has been used in traditional medicine for the treatment of a wide variety of medical conditions, including migraine, hypertension, bronchial asthma, and allergic rhinitis. This herb is primarily distributed in Europe, as well as in some regions of North America and Asia [2–4]. Different parts of the plant contain bioactive compounds with anti-inflammatory, spasmolytic, and antioxidant properties. The therapeutic effects of butterbur are principally due to secondary metabolites, primarily sesquiterpenes known as petasins, which are esters of petasol and angelic acid [5,6]. The concentration of petasins and their derivatives, specifically isopetasin, neopetasin, S-petasin, iso-S-petasin, neo-S-petasin, varies but is generally higher in the plant's rhizomes and roots. In addition to sesquiterpene esters, the rhizome and roots also contain toxic pyrrolizidine alkaloids (PAs). For this reason, extracts prepared from the plant for medicinal use must be free of PAs [2,6]. At present, the petasins are commercially available as dietary supplements for the prevention and treatment of migraine and allergic rhinitis [7–9]. It has been proposed that the mechanisms by which petasins exert anti-migraine and antiallergic effects are mediated by their anti-inflammatory activity and inhibition of leukotriene synthesis. However, calcium channel blockage by petasins stems from their spasmolytic potential and may also explain their anti-migraine action [10,11]. Although the exact modes of action of butterbur's compounds remain

elusive, their efficacy and favorable tolerance have been extensively documented [7,8,10]. In addition, petasins have shown efficacy against cardiovascular, gastrointestinal, and urogenital disorders and might be beneficial for the prevention of neurodegenerative diseases as well [2,11].

Recently, it was demonstrated that petasins and their derivates, isolated from different *Petasites* species, also exert anti-tumor activity. *In vitro* and some *in vivo* studies have shown strong antiproliferative activities of these bioactive substances towards various types of human cancers [12–16]. A new benzofuran derivative isolated from *P. hybridus* roots showed moderate inhibitory activity on human breast cancer MCF-7 cell proliferation [17,18]. Guo *et al*. [12] found that S-petasin from *Petasites japonicus* inhibited proliferation and migration of the human melanoma cell line A375, and induced apoptosis activating the tumor suppressor p53. Similarly, significant anti-tumor effects of petasin have been reported when different colorectal cancer cell lines were used. Furthermore, the authors of this study have also confirmed these effects in a murine colorectal tumor model showing reduced tumor growth. Moreover, findings suggested that petasin inhibited the Akt/mTOR signaling axis [13]. Another study revealed that exposure to S-petasin and iso-S-petasin isolated from common butterbur elicits high cytotoxicity and apoptotic cell death responses through caspase activation and cytochrome c release in prostate cancer cells [14]. Further, isopetasin and S-isopetasin extracted from *Petasites formosanus* have been shown to act as MDR inhibitors by targeting P-glycoprotein (P-gp) and suppressing the proliferation of multidrug-resistant cancer cells [15]. Our previous findings indicate that standardized *Petasites hybridus* L. root extract, with the active ingredient petasin and its derivatives, showed a selective cytotoxic effect towards non-invasive MCF-7 and highly invasive MDA-MB-231 breast cancer cells. These studies documented that MDA-MB-231 cells display an approximate two-fold higher sensitivity to this root extract [16]. However, the mechanisms that underlie the anti-breast cancer activities of sesquiterpene esters remain to be elucidated.

The aim of this study was to reveal the role of Bulgarian *Petasites hybridus* L. root extract on the induction of apoptosis, necrosis, oxidative stress, and NF-κB signaling when the highly invasive breast cancer MDA-MB-231 and non-cancerous MCF-10A cells were treated with this extract. The results indicated that standardized *Petasites* extract exhibited a selective pro-oxidant effect, increased NF-κB levels, and induced apoptosis in breast cancer cells whereas non-cancerous MCF10A cells remained only slightly affected. These findings suggest that the active compounds derived from the roots of *Petasites hybridus* L. may represent a promising anti-breast cancer therapy and that warrants further *in vivo* and clinical investigation.

2. Materials and Methods

2.1 Extract Preparation and Chromatographic Conditions for Petasin Analysis

A standardized butterbur powered extract containing a minimum of 15% petasins manufactured by Rumex Ltd., Sofia, Bulgaria (batch #321010) was used. The lipophilic extract was prepared from the subterranean portion of the plant, formally known as the radix petasitidis, from Bulgarian populations of *Petasites hybridus* L. These plant portions were extracted by maceration in ethanol, followed by liquid-liquid extraction with a more selective extractant. The crude extract was subjected to a final treatment in an aqueous medium pH value $\leq$4 for complete removal of the toxic pyrrolizidine alkaloids as determined by GH-MS method.

To prepare samples for High-performance liquid chromatography (HPLC) analysis of petasins, the mobile phase was used as a solvent. Analysis was performed on HPLC system Waters Alliance e2695 Separations Module with 4-channel degasser, Quaternary HPLC Pump, Autosampler with 100 µL loop, column thermostat and UV-VIS detector Waters 2998 PDA (Waters Corp., Milford, MA, USA). The analysis was performed on a Venusil XBP C 18 column (250 mm $\times$ 4.6 mm $\times$ 5 µm; Agela Tech, Torrance, CA, USA) with particle characteristics of 5 µm particle size, 150 Å pore size, and m^2/g surface area 200. The isocratic mode was used with mobile phase methanol, acetonitrile, and water (32:31:37, v/v/v) at 30 °C as described by Wildi *et al*. [19]. The eluent flow rate was 1.0 mL/min in isocratic mode. A content of the six main sesquiterpene esters was quantified versus an external petasin standard (Sigma-Aldrich, St. Louis, MO, USA) and we measured the peak area of sesquiterpene esters at 235 nm. Software for system control, data acquisition, and data processing was conducted using Empower 3 software, database v7.20 00 00 (Waters Corp., Milford, MA, USA).

For biochemical assays, the powdered extract was dissolved in fresh cell culture medium containing 2% dimethyl sulfoxide (DMSO) which was used as a solvent. The solution was filtered using 0.45 µm syringe filter prior to use. The final concentration of DMSO in the samples with different concentrations of *Petasites* extract was approximately 1%, which was shown to be completely non-toxic [16].

2.2 Cell Lines and Culture Conditions

The human breast cancer MDA-MB-231 and the non-transformed mammary epithelial cell line (MCF-10A) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The MDA-MB-231 cell line was cultured in complete Dulbecco's modified Eagle's medium (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (FBS), L-glutamine (2 mM) and Penicillin (100 U/mL)/Streptomycin (100 µg/mL)/Amphotericin B (0.25 µg/mL). The MCF-10A cell line was cultured in

Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (Sigma-Aldrich, USA), additionally supplemented with insulin (10 µg/mL), hydrocortisone (500 µg/mL), hEGF (20 ng/mL) and cholera toxin (20 ng/mL). Both cell lines were cultured in 37 °C and 5% CO_2 atmospheres. Both cell lines were authenticated by short tandem repeat profiling and were regularly tested for mycoplasma by using DAPI staining method and were confirmed negative for mycoplasma contamination.

2.3 Annexin V/PI Apoptosis Assay

Human breast cancer MDA-MB-231 cells and non-cancerous MCF-10A cells were plated in 100 mm petri dishes and T-25 flasks at a density of 5×10^5 and 3×10^5 cells, respectively. After a 24 h incubation, both types of cells were treated for 72 h with 260.4 µg/mL, 520.8 µg/mL and 781.2 µg/mL of *Petasites hybridus* L. root extract. These concentrations correspond to IC_{25}, IC_{50}, IC_{75} inhibitory concentrations that were previously empirically determined in MDA-MB-231 by MTT analysis [16]. To examine whether *Petasites hybridus* L. root extract elicits diverse responses in cancer and non-cancerous cells, MCF-10A cell line was treated with the same doses of plant extract. Untreated cells were used as a negative control. After treatment with the extract cells were centrifuged, washed with cold cell staining buffer, and resuspended in 100 µL of Annexin V binding buffer (Lot#B287709, BioLegend, San Diego, CA, USA). Following this, the cells were incubated with fluorescent allophycocyanin (APC) Annexin V (Lot#B278172, BioLegend) (5 µL) for 15 minutes in the dark. Finally, 1.5 µL of propidium iodide (PI) (Lot#B283508, BioLegend) and 400 µL Annexin V binding buffer were added and samples were analyzed using a FACSCalibur flow cytometry (Becton Dickinson, San Jose, CA, USA) and results analyzed using CellQuestPro software (Becton Dickinson, San Jose, CA, USA).

2.4 Measurement of Oxidative Stress Biomarkers

To evaluate the alterations in the redox balance and the occurrence of oxidative stress, the levels of glutathione (GSH) and malondialdehyde (MDA) were quantitatively estimated using cell lysates. The levels of GSH were measured by an enzyme-linked immunosorbent assay (ELISA) kit (Cat. No MBS161025, MyBioSource, Inc., San Diego, CA, USA), following the manufacturer's instructions. The absorbance was measured at a wavelength of 450 nm using a plate reader (Tecan Infinite F200 PRO (Tecan Austria GmbH, Salzburg, Austria)). MDA was measured colorimetrically by a thiobarbituric acid reactive substance (TB ARS) assay (Cat. No 10009055, Cayman Chemical, Ann Arbor, MI, USA), according to the manufacturer's instructions. The absorbance was measured at a wavelength of 530 nm using a plate reader (Tecan Infinite F200 PRO (Tecan Austria GmbH)).

2.5 Detection of Nuclear Factor kappa-B (NF-κB)

The quantification of the p105 subunit of human NF-κB in cell lysates was determined by ELISA (Cat. No, SEB824Hu, Cloud-Clone Corp., Katy, TX, USA) according to the manufacturer's instructions. The absorbance was measured at a wavelength of 450 nm using a spectrophotometer (Tecan Infinite F200 PRO (Tecan Austria GmbH)).

2.6 Statistical Analysis

All data in this study were analyzed using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, USA). Values are expressed as the mean ± standard deviation (SD) from three replicates. Significance testing was performed using one or two-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test or Bonferroni's post hoc test. Values of $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ were considered statistically significant.

3. Results

3.1 HPLC Analysis of Sesquiterpene Esters Isolated from Butterbur Powdered Extract

The principal active substances in *Petasites hybridus* L. are esters of 3-isomeric sesquiterpene alcohols, specifically petasole, iso-petasole and neopetasole. Active substances also include six sesquiterpene esters, specifically petasin and its isomers iso-petasin and neopetasin, and the methylthio derivative of petasin, s-petasin and its isomers iso-s-petasin and neo-s-petasin [6]. HPLC analysis of the investigated extract from the subterranean parts of *P. hybridus* (Fig. 1) show the presence of all six main sesquiterpene components with neopetasin, petasin and s-petasin being the dominate compounds in this extract.

3.2 Effect of Standardized Petasites hybridus L. Root Extract on the Induction of Apoptosis and Necrosis in Breast Cancer MDA-MB-231 Cells and Non-Cancerous MCF-10A Cells

To confirm and extend our previous findings demonstrating selective cytotoxicity and activation of apoptosis in breast cancer cells after exposure to *Petasites hybridus* L. root extract [16], cell death was further analyzed by Annexin V-APC/PI double stain flow cytometry. The stages of apoptosis and necrosis were monitored after treatment of breast cancer MDA-MB-231 cells and non-cancerous MCF-10A cells with different concentrations of the root extract of Bulgarian *Petasites hybridus* L. for 72 h. These concentrations correspond to the IC_{25}, IC_{50} and IC_{75} of *Petasites* extract determined in previous work [16]. The induction of apoptosis is a response often sought in cancer therapy, and the goal is to activate programmed death in cancer cells while minimizing this effect in normal cells [20,21].

The two cell lines were treated with the same increasing concentrations of the root extract of *Petasites hybridus* L., specifically, 260.4 µg/mL, 520.8 µg/mL and 781.2 µg/mL [16] and we monitored the toxic effect in can-

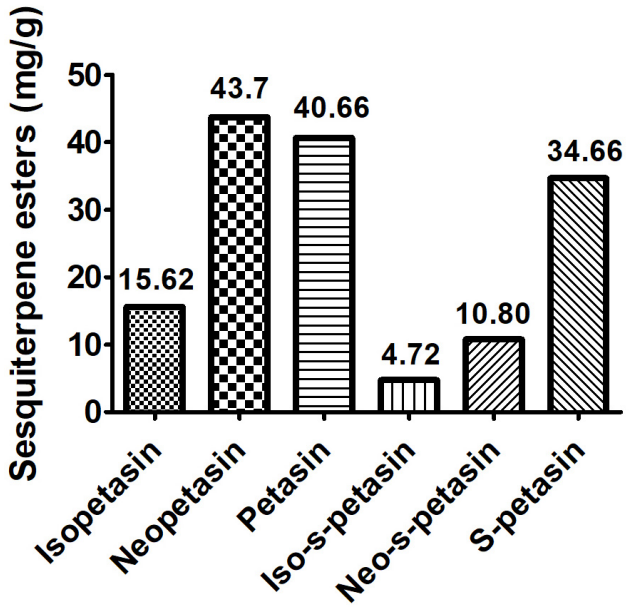

Fig. 1. HPLC analysis of sesquiterpene esters isolated from standardized 15% petasin *Petasites hybridus* L. root extract. The graph shows the concentration of the principal sesquiterpene esters in the extract expressed in mg/g. This equals the total amount of 15% petasins isolated from the powdered *Petasites hybridus* L. extract. Neopetasin, petasin and s-petasin are presented in relatively higher quantities when compared to the other identified sesquiterpene esters.

cer and non-cancer cells. In MDA-MB-231 cells, the percentage of early apoptotic cells, Annexin V-APC positive/PI- negative, was lower (4.41 $\pm$ 3.91% and 5.22 $\pm$ 3.13%), while the population of late apoptotic cells, Annexin V-APC and PI positive, was higher (13.05 $\pm$ 2.66% and 14.51 $\pm$ 0.71%) after treatment with IC_{50} and IC_{75} of *Petasites* extract, respectively (Fig. 2C). In contrast, the percentage of cells in early apoptosis was higher (13.33 $\pm$ 3.49% and 20.28 $\pm$ 7.67%) compared to those in late apoptosis (6.96 $\pm$ 2.81% and 5.49 $\pm$ 2.69%) in MCF-10A cells treated with IC_{50} and IC_{75} concentrations of *Petasites* extract (Fig. 2D).

The population of necrotic cells, Annexin V-APC negative/PI positive, remained relatively low in both cell lines (2.51 $\pm$ 1.92%; 2.65 $\pm$ 0.57% for MDA-MB-231 cells and 1.37 $\pm$ 1.12%; 0.90 $\pm$ 0.79% for MCF-10A cells) after treatment with IC_{50} and IC_{75} concentrations of *Petasites* extract. In general, the results show that the standardized *Petasites hybridus* L. root extract exerts differential apoptotic effects in cancerous and non-cancerous cells. An increase in the extract's concentration in MDA-MB-231 cells resulted in the development of late apoptotic cells, whereas in MCF-10A cells treated with the same concentration of *Petasites* extract resulted in a higher number of early apoptotic cells. This suggests an induction of a more robust apoptotic response in breast cancer cells compared to normal cells.

3.3 Induction of Oxidative Stress by the Standardized Petasites hybridus L. Root Extract

Oxidative stress is a potent inducer of apoptotic cell death [22]. In addition, several plant-derived bioactive compounds have been found to exert pro-oxidant activity resulting in increased reactive oxygen species (ROS) and selectively inducing cell death in cancer cells due to their excess ROS [23,24]. To test whether late apoptosis in breast cancer cells detected after treatment with *Petasites hybridus* L. extract was associated with an induction of oxidative stress, GSH and MDA were quantitatively measured as biomarkers of oxidative stress response. The reduced form of glutathione (GSH) is a major cellular antioxidant that neutralizes various forms of ROS, such as superoxide anion (O_2-•), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH•) [25]. MDA is a toxic aldehyde and is one of the final products of lipid peroxidation caused by excessive ROS and resulting cellular damage. An imbalance between the production of ROS and its elimination by antioxidant systems is referred to as oxidative stress [26]. Under normal physiological conditions, reduced GSH is maintained in high levels and thus effectively inhibits lipid peroxidation [27,28]. However, during an oxidative stress state, GSH levels are diminished which, in turn, elevates lipid peroxidation [29]. Hence, cellular GSH and MDA levels were measured after treatment of MDA-MB-231 and MCF-10A cells with standardized *Petasites hybridus* L. root extract at indicated concentrations for 72 h.

As shown in Fig. 3A, ELISA analysis determined that cellular GSH levels were significantly decreased in a concentration-dependent manner in MDA-MB-231 cells in the presence of increasing doses (IC_{50} and IC_{75}) of *Petasites* extract, when compared to untreated controls. In contrast, the treatment of MCF-10A cells, with the plant extract at these concentrations, led to a significant increase in GSH levels in a concentration-dependent manner (Fig. 3B).

To verify whether diminished GSH levels in MDA-MB-231 cells after exposure to *Petasites hybridus* L. root extract were related to a redox imbalance and induction of oxidative stress, the levels of MDA were also analyzed. As shown in Fig. 4A, MDA levels were slightly increased after exposure to the IC_{50} concentration and this value was significantly elevated when breast cancer cells were treated with IC_{75} when compared to non-treated controls. In MCF-10A cells, MDA levels remained elevated after treatment with *Petasites* extract with no rise in a concentration-dependent manner noted in Fig. 4B.

In sum, results obtained indicate that *Petasites hybridus* L. root extract acts as pro-oxidant, inducing oxidative stress specifically in MDA-MB-231 cells. In contrast, in non-cancerous MCF10A cells oxidative stress is alleviated due, in part, to a compensatory increase in the GSH antioxidant defense system. Therefore, these findings suggest that butterbur extract triggers a late apoptotic response in human breast cancer cells as result of oxidative stress,

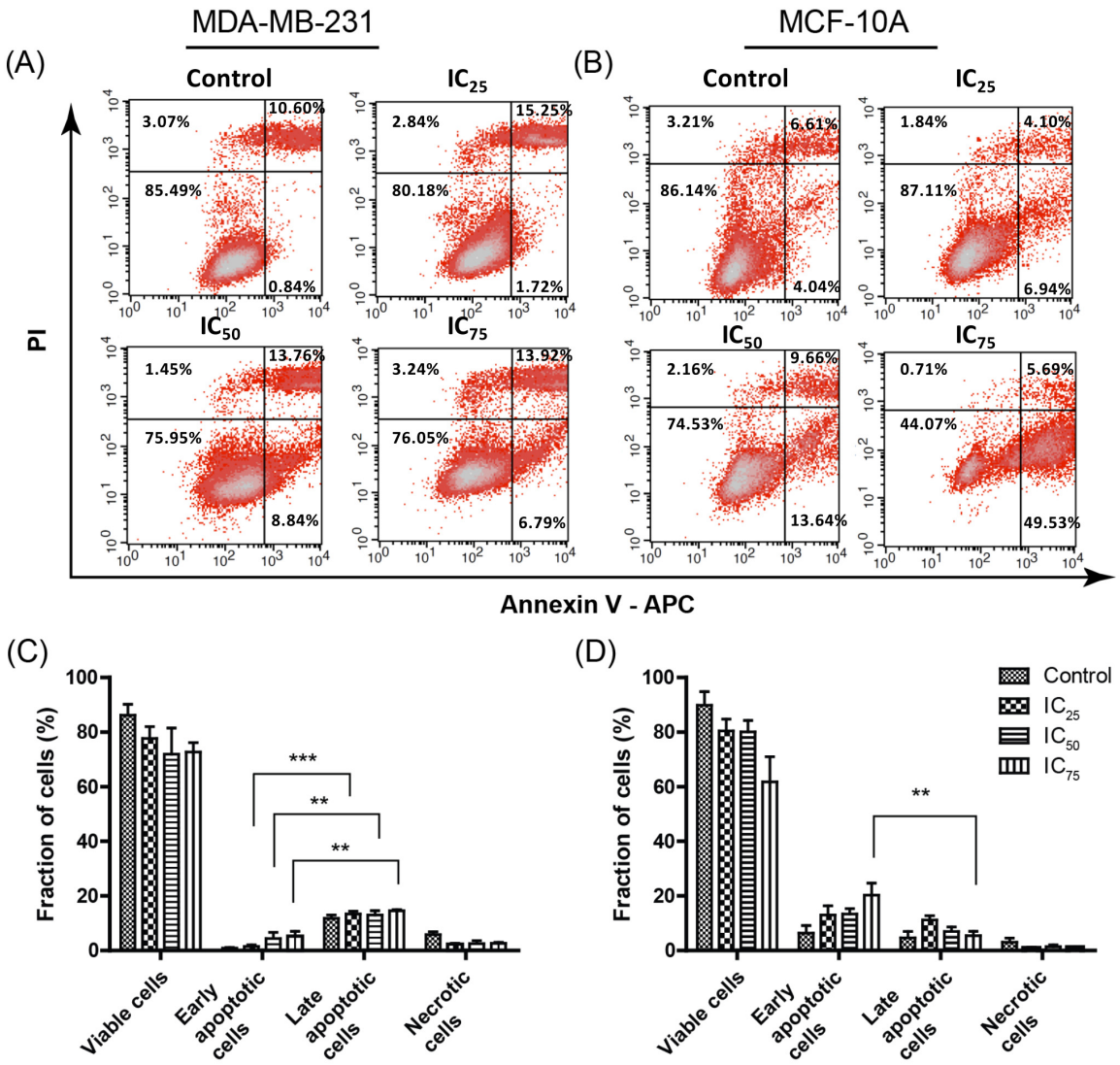

Fig. 2. Quantification of apoptosis in breast cancer MDA-MB-231 cells and non-transformed MCF-10A cells by standardized *Petasites hybridus* L. root extract. (A) and (B) Representative flow cytometry plots of apoptosis/necrosis in MDA-MB-231 and MCF-10A cells, respectively. Cells were treated for 72 h with 260.4 µg/mL (IC_{25}), 520.8 µg/mL (IC_{50}) and 781.2 µg/mL (IC_{75}) and stained with Annexin V-APC/Propidium iodide (PI) prior to analysis by flow cytometry. The four quadrants indicate viable cells (lower left), early apoptotic (lower right), late apoptotic (upper right) and necrotic cells (upper left). (C) and (D) Graphs illustrate the mean percentage of viable, early apoptotic, late apoptotic and necrotic cells in treated MDA-MB-231 and MCF-10A cells, respectively, from three independent experiments. The percentage of early apoptotic cells were statistically compared to late apoptotic cells and significance was determined by a two-way ANOVA with Bonferroni's post hoc test. Data are expressed as the mean ± SD of three independent experiments. ** $p < 0.01$ and *** $p < 0.001$.

and this response is accompanied by increased lipid peroxidation and GSH depletion.

3.4 The Standardized Petasites hybridus L. Root Extract Elevates NF-κB in MDA-MB-231 and MCF-10A Cells

To further investigate the link between nuclear factor kappa-B (NF-κB) and apoptosis in response to oxidative stress in breast cancer cells following exposure to *Petasites* extract, the abundance of NF-κB was measured using an ELISA assay. NF-κB is a multi-subunit transcription factor which plays essential roles in immune response, inflammation, cell proliferation, migration, and apoptosis [30]. Apart from its well-known anti-apoptotic role, NF-κB has also been found to exert pro-apoptotic functions, depending on the induced stress stimuli and the cell type. Under normal physiological conditions NF-κB is localized within the cytoplasm. However, in response to diverse stimuli such as inflammatory molecules, ultraviolet (UV) light, ionizing radiation (IR), and increased ROS, NF-κB is activated and undergoes translocation to the nucleus where it regulates

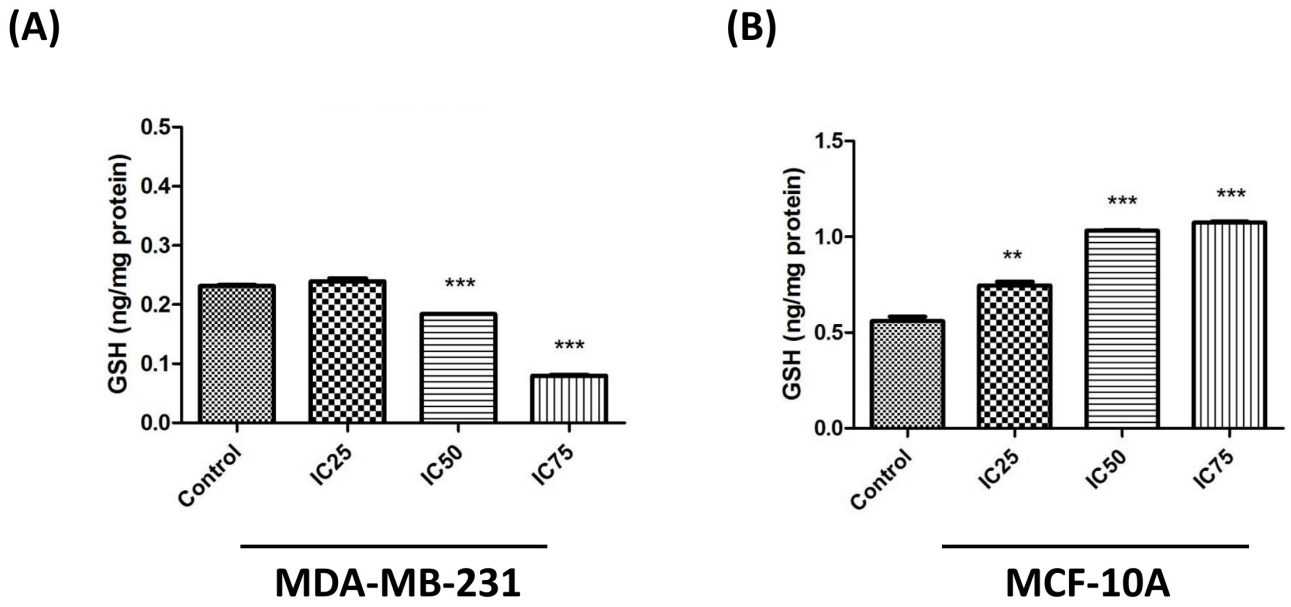

Fig. 3. The effect of standardized *Petasites hybridus* L. root extract on GSH levels in breast cancer MDA-MB-231 cells and non-cancerous MCF-10A cells. (A) and (B) GSH levels expressed as ng GSH/mg protein in MDA-MB-231 and MCF-10A after 72 h of treatment with 260.4 µg/mL (IC_{25}), 520.8 µg/mL (IC_{50}) and 781.2 µg/mL (IC_{75}) concentrations of the root extract. Non-treated cells from both types of cell lines were used as the control. GSH was measured by ELISA and data expressed as the mean ± SD of three independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to control (one-way ANOVA).

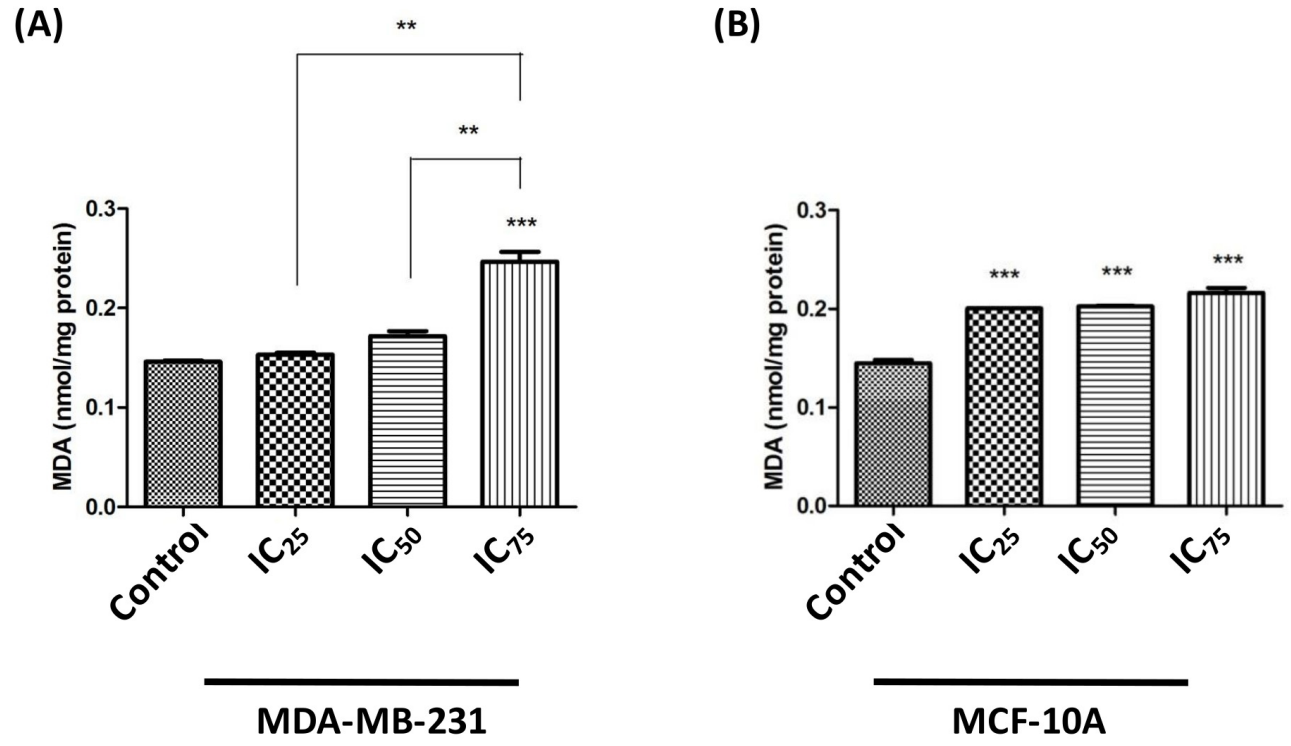

Fig. 4. The effect of standardized *Petasites hybridus* L. root extract on MDA levels in MDA-MB-231 and MCF-10A cells. (A) and (B) MDA levels expressed as nmol MDA/mg protein in MDA-MB-231 and MCF-10A after treatment of both cell lines for 72 h with 260.4 µg/mL (IC_{25}), 520.8 µg/mL (IC_{50}) and 781.2 µg/mL (IC_{75}) of the standardized plant extract. Non-treated cells from both cell lines were used as the control. The levels of MDA were measured by a TBARS assay kit and values represent the mean ± SD. Statistical significance was determined by a one-way ANOVA with Tukey's Multiple Comparison Test. $^{**}p < 0.01$ and $^{***}p < 0.001$.

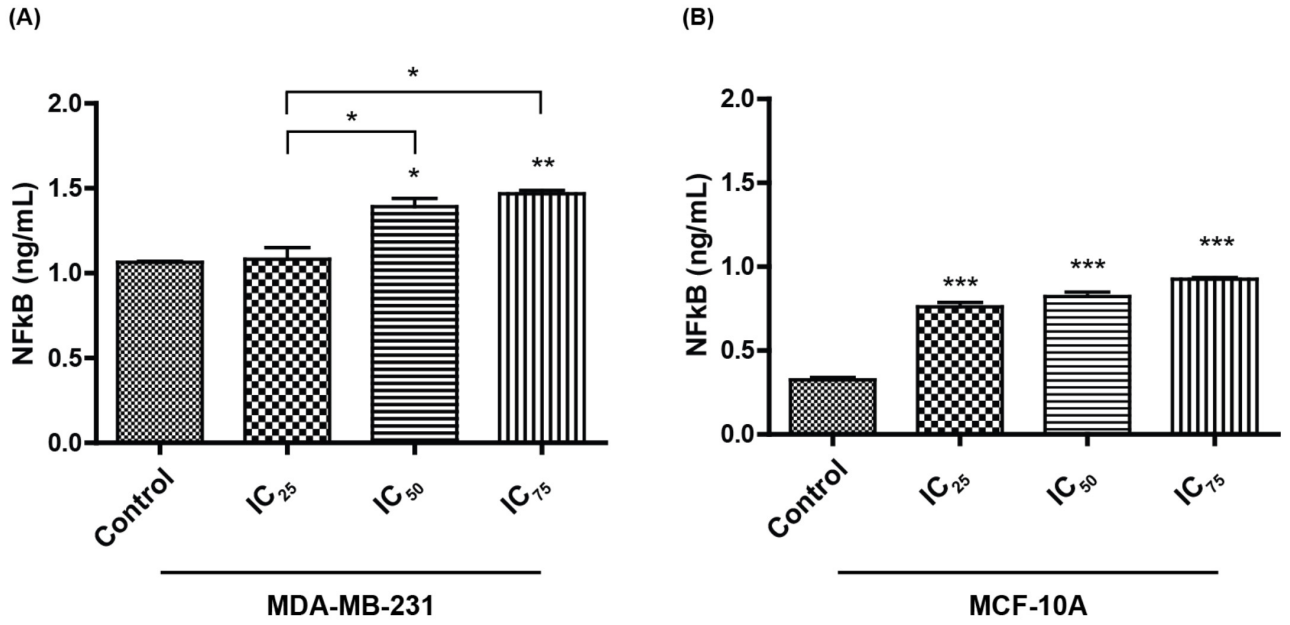

Fig. 5. The effect of standardized *Petasites hybridus* L. root extract on NF-κB levels in breast cancer MDA-MB-231 and non-cancerous MCF-10A cells. (A) and (B) NF-κB p105/p50 concentrations in MDA-MB-231 and MCF-10A after exposure to 260.4 µg/mL (IC_{25}), 520.8 µg/mL (IC_{50}) and 781.2 µg/mL (IC_{75}) concentrations of the root extract for 72 h. Non-treated cells from both types of cells were used as a control. NF-κB abundance was determined using a commercial ELISA kit from cell lysates developed from each cell lines. Results are expressed as means ± SD and analyzed by one-way ANOVA followed by Tukey's Multiple Comparison Test. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$.

downstream target gene expression [31,32]. Our results showed that *Petasites hybridus* L. root extract significantly increased NF-κB levels 72 h after treatment in both MDA-MB-231 and MCF-10A cells in a concentration-dependent manner (Fig. 5A,B). We found that the NF-κB abundance was higher in untreated MDA-MB-231 cells when compared to untreated MCF10A cells. This is very likely due to genetic and metabolic alterations in tumor cells which cause them to maintain elevated NF-κB levels in order to stimulate tumor cell proliferation and suppress apoptosis [33]. However, a further increase in NF-κB levels as a result of oxidative stimuli can mediate differing cell responses. For example, it has been documented that excessive ROS has a dual role in NF-κB signaling. Specifically, activated NF-κB can drive increased expression of pro-oxidant and pro-apoptotic genes; however, NF-κB activation also suppresses ROS by induction of antioxidant target genes [34]. Based on these findings, we propose that standardized *Petasites hybridus* L. root extract triggers NF-κB activation through the induction of oxidative stress, and that this response eventually provokes late apoptosis in MDA-MB-231 cells. In contrast, *Petasites hybridus* L. extract likely increases NF-κB in MCF-10A cells to mitigate oxidative stress through a compensatory increase of antioxidants and thus reduces the accumulation of cellular damage in healthy cells.

4. Discussion

Breast cancer is the second leading cause of death among women worldwide and, despite recent advances in breast cancer therapy, the mortality rate continues to rise [1]. Thus, new alternative approaches, with non or low-toxic treatment regimens, need to be developed. One principal therapeutic strategy is targeting apoptosis specifically within cancer cells and without harming normal cell function [35]. Apoptosis is a tightly coordinated process which is essential for the development and homeostasis of multicellular organisms. This type of regulated cell death efficiently eliminates harmful, damaged and malignantly-transformed cells [20,21]. In contrast to necrotic cell death, apoptosis does not cause inflammation in surrounding tissues, this feature makes induction of apoptosis in tumor cells a preferential cell response in cancer therapy. However, most currently used chemotherapeutics are highly toxic and induce apoptosis in both tumor and healthy cells causing systemic cellular injury.

Many plants and their bioactive compounds have long been the subject of study due to their multiple therapeutic properties. Such properties may make these compounds useful for the prevention and treatment of various diseases. Excluding toxic plant alkaloids that are currently used in cancer chemotherapy, the interest in herbal plant products with anti-proliferative activity and tolerability to normal cells has dramatically increased in recent years. A

growing body of evidence indicates that different plant-derived compounds such as polyphenols, flavonoids, phytosterols, and terpenoids (sesquiterpenes), exert anti-tumor effects through modulating diverse signaling pathways associated with cancer cell growth and invasiveness and, primarily, selectively inducing apoptosis [36–41]. We previously demonstrated selective cytotoxic effects and morphological changes associates with apoptosis in MCF-7 and MDA-MB-231 breast cancer cells after exposure to a standardized root extract of the medicinal plant *Petasites hybridus* L. with active sesquiterpenes termed petasins [16]. Common butterbur and its bioactive components are well-studied for treatment of migraine and other disorders, but their potential anti-tumor activity remains poorly investigated and available literature on this topic are quite limited [9].

Following the results of our previous work [16] which determined that the triple negative breast cancer cell line MDA-MB-231 displayed almost twice the sensitivity to common butterbur extract when compared to the MCF-7 cell line, in the present study we confirmed that standardized Bulgarian *Petasites hybridus* L. root extract triggered apoptosis in MDA-MB-231 cells after 72 hours of treatment. Moreover, Annexin V/PI flow cytometry showed that breast cancer cells were committed to late stages of apoptosis, whereas parallel treatment of non-cancerous MCF-10A cells displayed an early apoptotic response. Late stages of apoptosis are characterized by the formation of apoptotic bodies through a process dependent on activation of a class of proteases termed caspases. Caspase activation, in turn, commits the cell to a process of irreversible programmed cell death. In contrast, early apoptosis is a reversible process as studies have determined that early apoptotic cells can fully recover after the elimination of early apoptotic inducers [42,43]. Moreover, the phagocytosis of early apoptotic cells trigger the processes of regeneration and angiogenesis at sites of tissue injury suggesting that early apoptotic cell uptake is anti-inflammatory in nature and induces a tolerogenic response [44]. Meanwhile, neither MDA-MB-231 nor MCF-10A cells underwent necrosis after exposure to various doses of the extract, indicating that common butterbur selectively kills breast cancer cells through activation of programmed cell death. Other studies showed induction of late stages of apoptosis via different biological mechanisms when various cancer cells were exposed to purified petasin or its derivates [12–15]. Based on the findings outlined in this study, we propose that the pronounced pro-apoptotic effect of *Petasites hybridus* L. root extract on metastatic MDA-MB-231 is due to the presence of petasins.

A possible mechanism by which *Petasites hybridus* L. root extract induces apoptosis in cancer cells could be through increased production of reactive oxygen species (ROS). Under normal physiological conditions, ROS play an essential role in cellular metabolism and homeostasis. ROS levels are strictly regulated by the endogenous antioxidant defense system which principally comprised of superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH). Excessive ROS formation impairs redox homeostasis and leads to the occurrence of oxidative stress which is harmful to cellular macromolecules such as proteins, lipids, and DNA [45,46]. Typically, cancer cells display increased levels of ROS and, in order to maintain the redox homeostasis, they upregulate antioxidant defense systems. Several studies have demonstrated that ROS can induce cell proliferation [47], resistance to apoptosis [48], increased angiogenesis [49], and invasion and metastasis [50]. However, a further increase in ROS above certain thresholds stemming from either endogenous or exogenous sources disturbs redox balance in tumor cells resulting in oxidative stress [51,52]. Oxidative stress may trigger apoptosis by activating the apoptosome protein complex which initiates the caspase cascade resulting in activation of the intrinsic apoptotic pathway [46]. It should be noted that pro-oxidant agents can increase cellular levels of ROS to cytotoxic levels, and thus this type of agent may induce selective killing of cancer cells and therefore be therapeutically useful [23]. Abdelfatah *et al*. [15] previously documented that sesquiterpenes from *Petasites formosanus* act as ROS generators and can trigger a predominantly late apoptotic response in multidrug-resistant cancer cells. Additionally, several other lines of evidence demonstrated that different plant-derived compounds selectively induce apoptosis in cancer cells by driving ROS accumulation and further promoting oxidative stress [53]. Beyond their antioxidant capacities, in certain circumstances, various plant-derived products can exhibit a pro-oxidant role [54,55]. Accordingly, we showed that the standardized *Petasites hybridus* L. root extract disturbs the redox balance in MDA-MB-231 and promotes a state of oxidative stress as marked by enhanced MDA levels and depleted GSH levels. MDA, a common marker of oxidative stress, is an end-product of ROS and fatty acid interaction and results in lipid peroxidation and cell membrane damage [26]. GSH depletion is also indicative of oxidative stress [25] as this is the most abundant non-protein intracellular thiol and is commonly present in high (1–10 mM) concentrations. Reduced GSH abundance can have potential application as a marker for various human diseases [56].

The findings outlined in this study are consistent with previous literature regarding the selective pro-oxidant properties of other terpenoids and their ability to activate oxidative stress-mediated apoptosis in cultured human cancer cells [21,22,57]. In addition, the pro-apoptotic transcription factor NF-κB has been characterized as a cellular "sensor" for oxidative stress [58]. Thus, it is not surprising that we found elevated levels of the NF-κB (p105/p50) in breast cancer cells treated with *Petasites hybridus* L. root extract. Similarly, a previous study by Ricca *et al*. [59] demonstrated that overexpression of NF-κB promotes apoptotic cell death accompanied by ROS generation in

multidrug-resistant MCF-7 breast cancer cells. Another *in vitro* study determined that cisplatin, a well-characterized inducer of oxidative stress [60] and a commonly used first-line chemotherapeutic for various malignancies [61], promotes upregulation of NF-κB and induction of apoptosis in head and neck squamous cell carcinoma [62]. Cisplatin has been found to promote severe oxidative stress, associated with decreased antioxidant abundance and increased lipid peroxidation both of which can provoke injury to normal tissues as well [63,64]. In contrast, our finding determined that *Petasites hybridus* L. root extract blunts oxidative stress in healthy MCF-10A cells. Moreover, the present work confirmed that NF-κB activation is cell-specific [65], and that the protective effect of NF-kB can result in suppression of oxidative stress through increasing antioxidant abundance thus reducing cellular damage in non-cancerous cells.

Studies conducted in rats have indicated increased NF-κB activity and activation of pro-apoptotic proteins as a result of exogenously induced oxidative stress [66]. Although cancer is not the direct subject of this particular investigation, this study demonstrates the relationship between oxidative stress, NF-κB activation, and its pro-apoptotic role an *in vivo* setting. Several *in vitro* studies using human cancer cell lines have also confirmed the pro-apoptotic function of NF-κB following exposure to various stimuli. Stark *et al*. [67] observed that the nonsteroidal anti-inflammatory drug aspirin mediates apoptosis in colon cancer cells through activation of NF-κB. A similar report observed induction of apoptosis, that was NF-κB-dependent, after treatment of melanoma cells with a biotransformed soybean extract [68]. Moreover, a recent study suggested the involvement of NF-κB in activation of apoptotic cell death after exposure of MCF-7 breast cancer cells to anti-tumor tetrahydroquinolines [69]. However, further studies are required to clarify whether NF-κB is responsible for the initiation of the apoptotic pathway in MCF-7 cells.

Based on all these findings, which are in line with our study and that a pro-apoptotic role for NF-κB is observed in both *in vitro* and *in vivo* models, evidence suggests the potential use of *Petasites hybridus* L. root extract in anti-tumor therapy. However, further studies are needed to confirm NF-κB activation by documenting its translocation to the nucleus as well as elucidating the pathways that regulate this transcription factor and determining the signaling pathways that lie upstream of NF-κB.

5. Conclusions

In conclusion, our findings indicate that the Bulgarian standardized *Petasites hybridus* L. root extract induces oxidative stress and promotes a strong apoptotic response most likely, at least in part, as a result of elevated NF-κB levels in MDA-MB-231 metastatic breast cancer cells. The administration of parallel doses of extract affected non-cancerous MCF-10A cells to a lesser extent. Taken together, these results suggest that *Petasites hybridus* L. root extract might be useful in treatment of invasive breast cancers; however, further studies using *in vivo* tumor models and clinical trials are necessary to confirm these effects.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

Conceptualization — RT and LM; Methodology — VU; Software — IG; Validation — SA, TO and IG; Formal analysis — TO; Investigation — SA; Resources — TO; Data curation — IG; Writing—original draft preparation — SA; writing—review and editing — RT; Visualization — IG; Supervision — RT; Project administration — LM; Funding acquisition — LM. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

Given her role as Guest Editor, Rumiana Tzoneva had no involvement in the peer-review of this article and has no access to information regarding its peer-review. Full responsibility for the editorial process for this article was delegated to Amedeo Amedei. The authors declare no conflict of interest.

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