Flower extracts of *Filipendula ulmaria* (L.) Maxim inhibit the proliferation of the NCI-H460 tumour cell line

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ABSTRACT

Filipendula ulmaria (L.) Maxim (meadowsweet) is a popular medicinal species that can be found throughout most Europe and Asia. The plant is known for its rich antioxidants content, having compounds such as flavonoids and ascorbic acid. Therefore, the aim of this work was to investigate if the flower extracts of *Filipendula ulmaria* (L.) Maxim have cell growth inhibitory activity in three human tumour cell lines representative of non-small cell lung cancer (NCI-H460), melanoma (A375-C5) and breast adenocarcinoma (MCF-7). One of the most potent extracts was selected to be further studied in the NCI-H460 cells, by investigating its antiproliferative potential, the effect on cell cycle profile and on programmed cell death. The obtained results showed that all the extracts inhibited the growth of the mentioned cell lines, with the one obtained by decoction being the most potent. Its effect on the NCI-H460 cells was due to a reduction in cellular proliferation, but not to alterations in programmed cell death. Interestingly, cellular treatment with the extract caused a statistically significant increase in the cellular levels of p21. Data obtained highlight the potential interest of *F. ulmaria* as a source of bioactive compounds.

Keywords: *Filipendula ulmaria* (L.) Maxim; meadowsweet; tumour cell proliferation; p21.

1. Introduction

Plants have been used as traditional medicines throughout the centuries (Novais et al., 2004) and nowadays they still play an important role in human therapy, according to the World Health Organization (WHO, 2002). Indeed, in some Asian and African countries 80% of the population use traditional medicine (including plant based medicines) as primary health care (Panda, 2012).

Filipendula ulmaria (L.) Maxim, commonly known as meadowsweet, is an interesting example of useful plants reported by several ethnobotanical surveys and can be found not only in the Iberian Peninsula, but also in other regions of most of Europe and Western Asia, such as Poland and Russia (Barros et al., 2013). Homemade remedies prepared from this species have been described since the late 16th and 17th centuries. Sweet tea is made from the dried flower and has been used for supportive treatment of common cold, relief of minor articular pain (analgesic properties), or to facilitate renal and digestive elimination functions. After an evaluation of the pharmacologic effects, *in vitro* and animal studies suggest that it has anti-inflammatory/immunomodulatory, antibacterial and anticarcinogenic activities (Rauha et al., 2000; Churin et al., 2008; Yildirim and Turker, 2009; Nesterova et al., 2009; Cwikla et al., 2010)

To our knowledge, there are only two studies (not published in English), which have been carried out in order to investigate the tumour cell growth inhibitory activity of extracts from *F. ulmaria*. In one of those studies, carried out in Raji (Burkitt lymphoma cells), the authors state that ethanolic extracts of *F. ulmaria* were cytotoxic to those cells, at concentrations of 10 and 50 µg/ml (Peresun'ko et al., 1993). The other study indicates that the local administration of decoction flower extracts in mice with cervical dysplasia had beneficial effects in the frequency of squamous-cell carcinoma of the

cervix and vagina induced in mice by 7,12-dimethyl-benz(a)anthracene treatment (Spiridonov et al., 2005).

Plant material gathered in Portugal has revealed rich antioxidants content, having compounds such as phenolic compounds, α-tocopherol and ascorbic acid. Regarding the phenolic compounds, phenolic acids (caffeic acid derivatives and gallic acid), flavonols (glycosides of quercetin and isorhamnetin) and ellagitannins (digalloyl-hexahydroxydiphenol-glucose and trigalloyl digalloyl-hexahydroxydiphenol-glucose) have been described (Barros et al., 2013).

In the present work, the tumour cell growth inhibitory potential of *F. ulmaria* flower extracts was investigated in three different human tumour cell lines: NCl-H460 (non-small cell lung cancer), A375-C5 (melanoma) and MCF-7 (breast adenocarcinoma). The most potent extract was further studied regarding its effect in cellular proliferation, cell cycle and programmed cell death.

2. Material and Methods

2.1. Samples and preparation of the extracts

The top 20 cm of the flowering stems and inflorescences with flowers fully open and functional of *Filipendula ulmaria* (L.) Maxim were collected in the Natural Park of Montesinho territory (Trás-os-Montes, North-eastern Portugal) in May 2009. Voucher specimens are kept at the Herbário da Escola Superior Agrária de Bragança (BRESA). The samples to be studied were lyophilized (Ly-8-FM-ULE, Snijders, Netherlands) and further submitted to four different extraction methodologies (**Table 1**).

For infusion preparation the lyophilized material (1 g) was added to 200 ml of boiling distilled water and left to stand at room temperature for 5 min, and then filtered under reduced pressure.

For decoction preparation the lyophilized material (1 g) was added to 200 ml of distilled water, heated (heating plate, VELP scientific) and boiled for 5 min. The mixture was left to stand for 5 min and then filtered under reduced pressure. The obtained infusion and decoction extracts were frozen and lyophilized.

For methanol and methanol:water (80:20 *v:v*) extracts preparation the lyophilized material (1 g) was extracted twice by stirring (25 ºC at 150 rpm) with 30 ml of methanol or methanol:water 80:20 (*v:v*), respectively, for 1 h and subsequently filtered through a Whatman No. 4 paper. The methanolic extracts were evaporated at 40 ºC (rotary evaporator Büchi R-210, Flawil, Switzerland) to remove methanol. In the case of methanol:water (80:20 *v:v*) extract, the aqueous phase was lyophilized.

Stock solutions of all extracts were prepared in DMSO at a concentration of 100 mg/ml and further stored at -20ºC.

2.2. Cell culture

The human tumour cell lines used in the study were: NCl-H460 (non-small cell lung cancer, a kind gift from NCl, USA), A375-C5 (melanoma, ATCC) and MCF-7 (breast adenocarcinoma, ATCC). Cells were maintained in RPMI-1640 medium with ultraglutamine (Lonza, Pittsburgh, PA, USA) supplemented with 5% FBS (Gibco, Alfragide, Portugal), at 37ºC in a humidified incubator with 5 % CO_2. All the experiments were performed with exponentially growing cells presenting more than 90% cell viability.

2.3. Cell growth inhibition assay

Cells were plated in 96-well plates at their previously determined optimal concentrations and incubated for 24 hours to adhere. In the case of NCI-H460 and

MCF-7 cells, the appropriate density was $5.0x10^4$/ml, and in the case of A375-C5 cells the appropriate density was $7.5x10^4$/ml. The sulforhodamine assay was adapted from the procedure used in the NCI's *in vitro* anticancer drug screening (Vichai and Kirtikara 2006; Neves et al.; 2011). Briefly, cells were treated with five serial dilutions of each extract, ranging from 25 µg/ml to 400 µg/ml. Following 48 h incubation with the extract (or immediately for the T0 plate), plates were fixed by adding ice-cold 10 % TCA (w/v, final concentration, Panreac, Barcelona, Spain) and stained with 1% SRB (Sigma Aldrich, St. Louis, MO, USA) in 1% (v/v) acetic acid. Bound dye was solubilised by adding 10mM Tris base solution (Sigma Aldrich, St. Louis, MO, USA) and finally the absorbance was measured at 510 nm in a microplate reader (BioTek® Synergy HT, Winooski, VT, USA).

2.4. Preparation of cells for other analysis

For the analysis of cellular proliferation, NCI-H460 cells were plated at $1.5x10^5$ cells/well in 6-well plates and incubated for 24 h. Cells were then treated with extract 2 (obtained by decoction) at its GI_{50} and 2 x GI_{50} concentrations. Blank cells (treated with medium) or control cells (treated with DMSO, equivalent to the GI_{50} and 2 x GI_{50} of the extract B) were also included. Cells were then analysed for cellular proliferation, cell cycle profile, programmed cell death or protein expression. The percentage of viable cells in relation to Blank cells was also confirmed, by trypan blue exclusion, 48 h after the addition of the extracts to the cells.

2.5. Cell proliferation analysis

Cellular proliferation was analysed according to the protocol previously described (Palmeira et al., 2010). Briefly, following 47 h incubation, cells were treated with 10

μM BrdU (Sigma Aldrich, St. Louis, MO, USA) for 1 hour. Cells were then harvested and fixed in 4 % paraformaldehyde (Panreac, Barcelona, Spain) in PBS for 30 minutes at room temperature. Following centrifugation, cell pellets were re-suspended in PBS and stored at 4ºC until processed. Cell cytospins were prepared and incubated in 2 M HCl (Sigma Aldrich, St. Louis, MO, USA) for 20 minutes. Following incubation with mouse anti-BrdU antibody (diluted 1/10, Dako, Glostrup, Denmark) for 1 hour at room temperature, cells were further incubated with anti-mouse-Ig-FITC (diluted 1/100, Dako, Glostrup, Denmark) for 30 minutes, at room temperature. Slides were then mounted with Vectashield mounting medium (with DAPI). BrdU incorporation was analysed using a fluorescence microscope (Nikon eclipse E400 microscope, Melville, NY, USA) and a semi-quantitative evaluation was carried out by counting a minimum of 500 cells per slide.

2.6. *Analysis of cell cycle distribution profile*

Following 48 h treatment, cells were fixed with ice-cold 70% ethanol (Fisher Scientific, Porto Salvo, Portugal) and stored at 4ºC for at least 12 h. Prior to analysis, cells were re-suspended in PBS containing RNase A (0.1 mg/mL) (Sigma Aldrich, St. Louis, MO, USA) and propidium iodide (5 µg/mL) (Sigma Aldrich, St. Louis, MO, USA). Cellular DNA content was analysed by flow cytometry (Becton Dickinson FacsCalibur Flow cytometer, Billerica, MA, USA) and the percentage of cells in the G1, S and G2/M phases of the cell cycle (as well as the percentage of cells in the sub-G1 peak) were determined using the FlowJo 7.6.5 software (Tree Star, Inc., Ashland, OR, USA) after cell debris and aggregates exclusion and plotting at least 20000 events *per* sample (Vasconcelos et al., 2000).

2.7. *Analysis of programmed cell death*

Following 48 h of treatment, cell pellets were fixed with 4 % paraformaldehyde in PBS and further stored in PBS at 4ºC. TUNEL assay was carried out using the "*in situ* cell death detection kit – fluorescein" (Roche, Amadora, Portugal) according to manufacturer´s instructions. Briefly, cell cytospins were incubated in an ice cold "Permeabilization Solution" (0.1% Triton®X-100 in 0.1% sodium citrate, Promega, Madison, USA) for 2 minutes and then incubated with TUNEL reaction mix (1:20 enzyme dilution, Roche, Amadora, Portugal) for 1 hour at 37ºC. Slides were mounted with Vectashield mounting medium (with DAPI). Cells were analysed using a fluorescence microscope and semi-quantitative evaluation of cells undergoing programmed cell death was assessed by counting a minimum of 500 cells per slide (Lima et al. 2004; Queiroz et al., 2013).

2.8. Protein expression analysis

Following 48 h of treatment, cell pellets were lysed in Winman's buffer (1% NP-40, 0.1 M Tris -HCl pH 8.0, 0.15 M NaCl and 5 mM EDTA) complemented with protease inhibitor cocktail (Roche, Amadora, Portugal). The total protein content was quantified using the DC™ Protein Assay kit (Biorad, Hercules, CA), according to manufacturer's instructions. Proteins (20 µg) were loaded on 12% SDS-PAGE gel and transferred into a nitrocellulose membrane (Amersham, Pittsburgh, PA, USA). The following primary antibodies were used: mouse anti-p53 (1:200, Thermo Scientific), mouse anti-p21 (1:250, Calbiochem Darmstadt, Germany, rabbit anti-p-H2A.X (1:200, Santa Cruz Biotechnology, Heidelberg, Germany) and goat anti-Actin (1:2000, Santa Cruz Biotechnology, Heidelberg, Germany). The corresponding secondary antibodies were: anti-mouse IgG-HRP (1:2000, Santa Cruz Biotechnology, Heidelberg, Germany), anti-rabbit IgG-HRP (1:2000, Santa Cruz Biotechnology, Heidelberg, Germany) or anti-goat IgG-HRP (1:2000, Santa Cruz Biotechnology, Heidelberg, Germany). The Amersham™ ECL Western Blotting Detection Reagents (GE Healthcare, Cleveland, Ohio, USA), the Amersham Hyperfilm ECL (GE Healthcare) and the Kodak GBX developer and fixer (Sigma, St. Louis, MO, USA) were used for signal detection, as previously described (Lima et al., 2006).

2.9. Statistical analysis

The results of the four tested extracts were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's HSD. In the remaining cases, results were analyzed by applying a Student's t-test (paired test). All tests were carried out using SPSS v. 20.0 program (SPSS Inc.), with $P \leq 0.05$.

3. Results and Discussion

3.1. Effect of the flower extracts on tumour cell growth inhibition

All the extracts studied inhibited cell growth in the cell lines studied. Results obtained for the GI_{50} concentrations (which inhibit cell growth by 50%) indicate that the most potent extract for the NCI-H460 and for the A375-C5 cells was extract 2 (obtained by decoction). In the MCF-7 cell, extracts 2, 3 and 4 presented similar effect and were slightly more potent than extract 1 (**Table 2**).

These results are in agreement with the ones obtained by the Russian study in the Raji cell line, in which extracts of *F. ulmaria* presented cytotoxic effects (Peresun'ko et al., 1993). Nonetheless, the mechanisms behind such an effect are still not known. Therefore, extract 2 (obtained by decoction) was selected to be further tested in the NCI-H460 cell line. This cell line was selected for being a cell line representative of lung cancer, one of the most mortal types of cancer.

3.2. Effect of extract 2 on NCI-H460 cellular proliferation

The effect of this extract on the proliferation of NCI-H460 cells was assessed with the BrdU incorporation assay. Results clearly show that this extract reduces cellular proliferation. Nevertheless, the effect was most evident only with the $2xGI_{50}$ concentration (**Table 3**).

The lower effect on cellular proliferation obtained with the GI_{50} concentration could be justified by the fact that the SRB assay is based on the protein cellular content and not on cell number. Indeed, when the effect of the same concentrations of extract was studied regarding viable cell number, it was possible to conclude that the previously determined GI_{50} concentration did not reduce the viable cell number but that the $2xGI_{50}$ concentration reduced viable cell number to less that 50% (**Figure 1**). This is probably

because a reduction in cellular protein content does not necessarily represent a reduction in cellular viability, at least not at the same time. This situation was already reported in similar studies with other natural matrices previoulsy carried out by some of the authors (Santos et al., 2013).

3.3. Effect of extract 2 on NCI-H460 cell cycle profile

Considering the reduction in cellular proliferation observed, the effect of the extract on cell cycle profile was investigated. Results (**Figure 2**) indicate that treatment with the $2xGI_{50}$ concentration of the extract caused a slight increase in the G1 phase of the cell cycle but this was not considered statistically significant. Nonetheless, considering the previously obtained results in cellular proliferation, an increase in the number of cells in the G1 phase of the cell cycle would make sense. In fact, cell cycle arrests cause decrease in cellular proliferation (Chen et al., 2010).

3.4. Effect of extract 2 on NCI-H460 programmed cell death

The effect of this extract on programmed cell death was also analysed with the TUNEL assay. Results show that the extract did not cause any increase in programmed cell death, at the concentrations and time point analysed (**Table 4**).

3.5. Effect of extract 2 on the expression levels of proteins involved in cellular proliferation and DNA damage, in NCI-H460 cells

Given the previous results, which indicated that the $2xGI_{50}$ concentration of this extract reduced cellular proliferation and caused a slight alteration of the cell cycle profile at the studied time point, the effect of this concentrations and duration of treatment on the expression of cellular proteins involved in cell cycle was investigated. Results from

Western Blots clearly showed that this treatment caused a statistically significant increase in the cellular levels of p21, and a non-statistically significant increase of p53 (**Figure 3**).

The increase in p21 is particularly interesting, considering the indication that there was also a small increase in the number of cells in the G1 phase of the cell cycle, since p21 is a G1-checkpoint cyclin-dependent kinase inhibitor (Abukhdeir and Park, 2008). It is well known that p21 expression is driven by p53 (Kuribayashi and El-Deiry, 2008) and therefore the increase in p53 found in our study could be the cause for the p21 increase. Nonetheless, it is known that the p21 tumor suppressor not only plays a role in p53 signaling but interestingly also positively regulates p53 expression (Mirzayans et al., 2012).

As p53 also controls apoptosis, alterations in the programmed cell death levels of treated cells would be expected but were not found in the present study. It is possible that if the study was extended to other concentrations and times of treatment, alterations in cell death would also be observed.

Since it is established that p53 expression could be increased as a result of DNA damage in wild-type p53 cells (Kuribayashi and El-Deiry, 2008), the present study was extended to look at the cellular protein levels of p-H2A.X following treatment with the extract. An increase in p-H2A.X levels was observed, even though this was not statistically significant, which is a possible indication of DNA damage (Tanaka et al., 2006). These results will need to be further confirmed with other specific assays for DNA damage. In fact, such a result is surprising considering the fact that the flowers from this plant are known for their rich antioxidants content (Barros et al., 2011; Barros et al., 2013). Nevertheless, those antioxidants might not be enough to avoid DNA damage, as least under the tested *in vitro* conditions.

It would be interesting to isolate the compounds present in extract 2 of *F. ulmaria* and identify which one(s) are causing the inhibition of proliferation effect in the tumour cells.

Overall, flower extracts of *F. ulmaria* inhibit the growth of the studied human tumour cell lines. The most potent extract was the one obtained by decoction, whose effect on the NCI-H460 cells was due to a reduction in cellular proliferation, causing an increase in the cellular levels of p21. Data obtained highlight the potential interest of this plant as a source of bioactive compounds.

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Table 1. Methods of preparation of the extracts from *Filipendula ulmaria.*

Extract number	Extraction method [a)]
1	Infusion
2	Decoction
3	Methanol extaction
4	Methanol:water 80:20 (*v:v*) extraction

[a)]Stock solutions of all extracts were prepared at 100mg/ml.

Table 2. Determined GI_{50} concentrations of the *Filipendula ulmaria* flower extracts.

Extract	NCI-H460	MCF-7	A375-C5
1	123.3 ± 5.4^{a}	113.3 ± 7.2^{a}	79.7 ± 6.2^{b}
2	70.0 ± 8.6^{c}	96.0 ± 12.4^{b}	63.3 ± 7.6^{c}
3	121.9 ± 17.7^{a}	91.5 ± 13.8^{b}	89.7 ± 12.8^{a}
4	79.7 ± 3.6^{b}	96.3 ± 14.2^{b}	83.7 ± 6.9^{a}

Values correspond to the mean ± S.E. of three independent experiments, carried out with duplicates. In each column different letters mean significant differences ($p<0.05$).

Table 3. Percentage of NCI-H460 cellular proliferation following treatment with extract 2, obtained by decoction of *Filipendula ulmaria* flowers.

Treatment	% BrdU positive cells
Blank	27.2 ± 3.5
DMSO	31.2 ± 2.8
2xDMSO	27.2 ± 3.5
GI_{50}	23.9 ± 1.8
$2xGI_{50}$	16.5 ± 2.9

Results are the mean ± S.E. of three independent experiments.

Table 4. Percentage of NCI-H460 cells undergoing programmed cell death following treatment with extract 2, obtained by decoction of *Filipendula ulmaria* flowers.

Treatment	% of cells undergoing programmed cell death
Blank	2.9 ± 0.1
DMSO	2.0 ± 0.4
2xDMSO	1.5 ± 0.1
GI50	2.0 ± 0.3
2xGI50	2.5 ± 0.1

Results are the mean ± S.E. of three independent experiments.

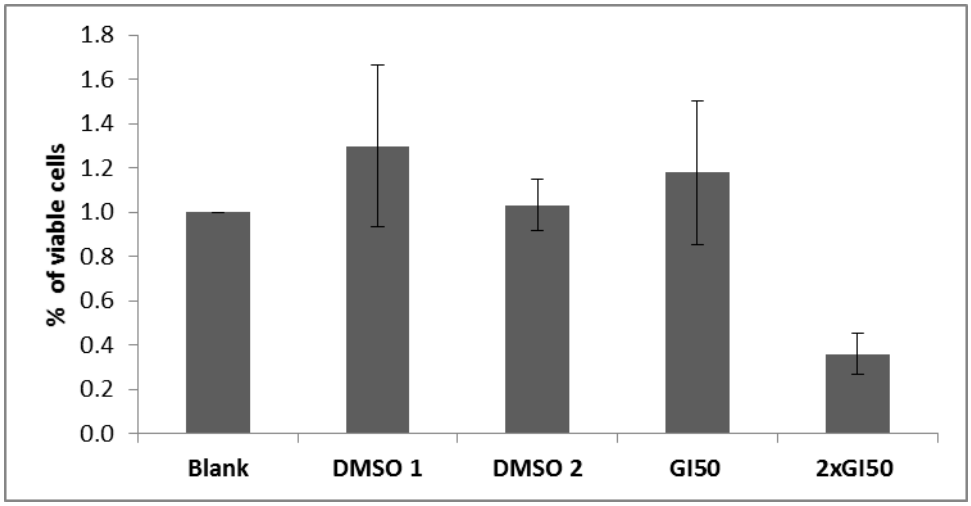

Figure 1. **Effect of extract 2 on NCI-H460 viable cell number.**

Cells were treated for 48 h with medium (Blank), extract (GI_{50} and $2xGI_{50}$) or with the corresponding DMSO volumes (DMSO 1 and DMSO 2, respectively). Viable cell number was assessed with the Trypan blue exclusion assay. Results are the mean ± SEM of 3 independent experiments.

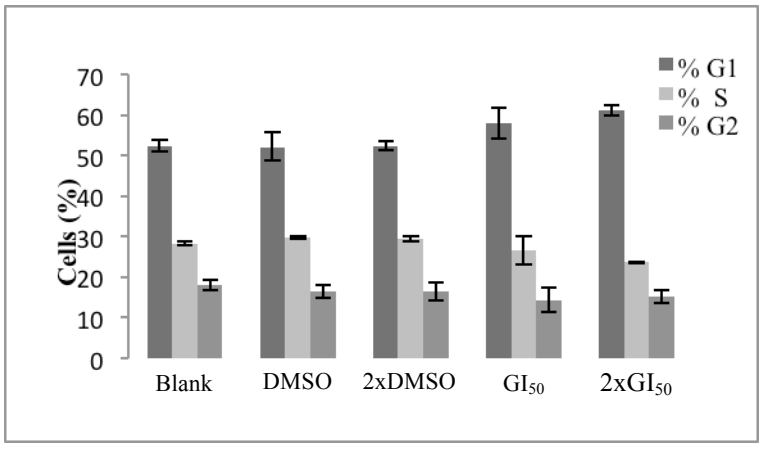

Figure 2. **Effect of extract 2 on NCI-H460 cell cycle profile**.

Cells were treated for 48 h with medium (Blank), extract (GI_{50} and $2xGI_{50}$) or with the corresponding DMSO volumes (DMSO and 2xDMSO, respectively). Results are the mean ± SE of 3 independent experiments.

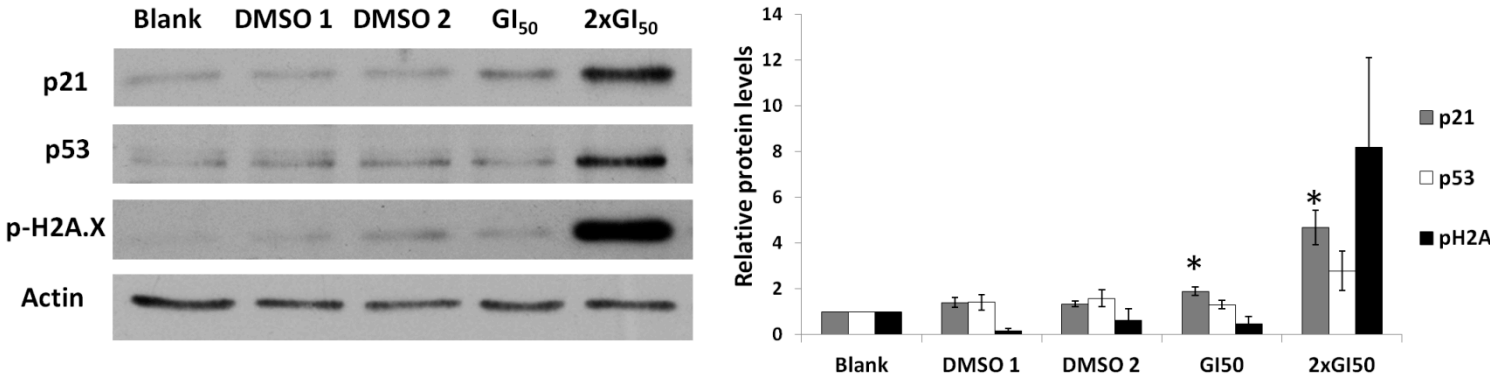

Figure 3. Effect of extract 2 on the expression levels of proteins involved in cellular proliferation and DNA damage, in NCI-H460 cells.

Cells were treated for 48 h with medium (Blank), extract (GI_{50} and $2xGI_{50}$) or with the corresponding DMSO volumes (DMSO 1 and DMSO 2, respectively). Actin was used as loading control. Left panel shows a representative image of Western blot. Right panel shows the results of densitometric analysis expressed after normalization of the values obtained for each protein with the values obtained for actin and further expressed in relation to blank cells. Results are the mean ± SE of 3 independent experiments. * $P \leq 0.05$ between treatment and Blank.