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RESEARCH ARTICLE

Identification of Bioactive and Anticancer Properties of Bidens Pilosa *in-vitro* Evidence

Walid Said[1], Abeer Ahmed Khattab[1], Saadia Aly Hamed[1], Sabah Alsayed Abo-Elmaaty[1], Hany Khalil[2]*

Abstract

Objectives: *Bidens pilosa* and *Trianthema portulacastrum* are noteworthy weeds with a series of bioactive flavonoid constituents, hence, they can be utilized as potential health supplements and readily available sources of natural antioxidants, as well as effective constituents in medicinal applications. The current study aims to assess the anti-proliferative activity of *B. pilosa* and *T. portulacastrum* extracts using the HepG2 cell line. Methods The prepared extracts were evaluated for their cytotoxic influence and their potential CC_{50} in HepG2 cell lines and normal hepatocytes using the MTT assay. Using quantitative real-time polymerase chain reaction (qRT-PCR), the relative gene expression of *Raf-1*, *MEK-1, LC3B*, and *Atg12* was quantified in treated cells to detect the expression levels of cell proliferation factors and autophagy-related genes. The quantification analysis of the released interleukin-1beta (IL-1β) and interleukin-1alpha (IL-1α) was also done using an ELISA assay. **Results:** The activities of *B. pilosa* extract showed an anti-proliferative influence on HepG2 cell lines upon treatment as compared to normal cells. It was assessed for cytotoxicity using molecular studies against both Raf-1 and *MEK-1* as proposed anticancer mechanisms and showed promising inhibitory activity against Raf-1 and *MEK-1* gene expression. Likewise, the reduction of autophagy-related genes, *Atg12* and *LC3B*, in HepG2 cells pre-treated with *B. pilosa* extract, further confirmed its influence in the induction of programmed cell death (PCD). The ELISA assay revealed a substantial elevation of the pro-inflammatory cytokines *IL-1α* and *IL-1β* upon treatment. **Conclusion:** This study found that *B. pilosa* extract, without any detectable cytotoxic effects, had potential inhibitory activities against both *Raf-1* and *MEK-1* gene expression, and a significant reduction in autophagic machinery upon treatment.

Keywords: Hepatocellular carcinoma- Ras/Raf/MEK/ERK signaling pathway- Autophagy- Cytokines (*IL-1α, IL-1β*),

Introduction

Hepatocellular carcinoma (HCC) is the most prevalent malignancy and one of the leading causes of primary cancer-related death worldwide. The global incidence and mortality rates of HCC are approximately equal [1]. Current HCC therapy options, inclusive of surgical procedures, locoregional ablative techniques, and interventional ablation treatments, have the potential to boost the 5-year survival rate to 75% (vs. 30% prior to these treatments). However, only 20% of HCC patients qualify for these treatments [2]. The scientists are facing crucial innovations for treating of intermediate and advanced stages of HCC after decades of frustrating nihilism owing to a lack of creative therapeutic solutions. Natural-derived substances are attracting scientific and academic attention since they are thought to have fewer hazardous side effects than conventional treatments such as chemotherapy. Current recommendations for HCC recognition, endorsed by professional society guidelines, include semi-annual abdominal ultrasound, with or without serum alpha-fetoprotein (AFP), in patients with cirrhosis and subgroups with chronic hepatitis B virus infection. HCC screening is supported by limited randomized clinical trial data from Asia among patients with chronic hepatitis B virus infection and numerous cohort studies among patients with cirrhosis. These studies consistently demonstrate that screening is significantly associated with early HCC detection, increased curative treatment receipt, and improved survival [3]. Biomarkers of the carcinogenic process encompass abnormal protein signaling pathways leading to uncontrolled cell proliferation, differentiation, survival, and apoptosis. The Ras/Raf/MEK/ERK cascade reaction is crucial for signal transduction pathway integration and is also related to cell cycle control, apoptosis, and cell differentiation [4–6]. The cytokine IL-1 has long been regarded as an effective modulator of immunity and inflammation. Its dysregulation has recently been associated with carcinogenesis and tumor progression,

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and its upregulation is anticipated to be correlated with a variety of malignancies. The IL-1 agonists IL-1α and IL-1β have been proven to enhance tumor invasiveness and metastasis by improving the production of angiogenic factors and growth factors [7]. Autophagy serves a crucial role not only in normal liver physiology, such as misfolded protein clearance and nutrient and energy metabolism in hepatocytes, but also in the pathogenesis of liver diseases such as non-alcoholic and alcoholic fatty liver, drug-induced liver injury, protein conformational liver diseases, viral hepatitis, fibrosis, liver cancer, cardiac diseases, and ageing [8, 9]. Autophagy and apoptosis are genetically-regulated biological mechanisms that control cell division and survival and are vital in maintaining cell fate. Manipulation of the autophagy system may be the key to controlling malignant cell behavior and inducing apoptosis [10, 11].

On the other hand, medicinal plants are prospective sources of bioactive phytochemicals, particularly phenolics, that have long been recorded as anticancer agents [12–14]. Bidens pilosa L. (Asteraceae), is an annual, easy grown, and widespread herb throughout the tropical and sub-tropical regions of the world. *Trianthema portulacastrum L.* (Aizoaceae) is an annual or perennial fleshy herb (based on geographical area), widespread in agricultural fields, especially in rainy seasons. Both weeds are exceptional sources of a diverse active principles, notably flavonoids, which have natural antimicrobial, anti-inflammatory, antioxidant, anticancer and other bioactivities [15]. *B. pilosa* has a long history of use in ethno-medicine to treat gastrointestinal and liver ailments, hence it has been recorded as a hepatoprotective and cytotoxic agent against various cancer cells [16]. *T. portulacastrum* is efficacious against heart diseases, inflammation, piles, ascites, alcohol poisoning, and toxin-induced hepatocarcinogenesis [17]. Accordingly, in the current study we aimed to investigate and compare the potential anti-proliferative effects of *B. pilosa* and *T. portulacastrum* extract on HepG2 cell line and identify the possible proliferation effector that might be involved in their influence.

Materials and Methods

Plant material and extraction

Fresh leaves of *B. pilosa* and *T. portulacastrum* were collected from Qanatir horticulture research institute fields in Qalyubia (Lat. 30° 10` 56``, Long. 31° 07` 50.7``) during summer 2020. The obtained samples were sterilized with 75% ethanol and ground with liquid nitrogen, then 10 mg of the ground powder was dissolved in 1 mL of DMSO to achieve a final concentration of 10 mg/mL. The final extract was kept at 4°C for further use.

Cell lines

The HepG2 cell line (The Egyptian holding company for biological products and vaccines, VACSERA, Dokki, Egypt) was cultured in RPMI media containing 4 mM L-glutamine, 4 mM sodium pyruvate, and 5% heat-treated bovine serum albumin (BSA). Normal hepatocytes were cultured in RPMI media containing 4 mM L-glutamine

and 10% BSA. All cell lines were incubated at 37°C in a 5% CO_2 condition [18]. Inverted microscope (Zeiss A-Plan 10X) was used to image grown cells. Cells were routinely checked examined for mycoplasma contamination.

Cytotoxic concentration of 50% (CC$_{50}$)

The obtained extracts were evaluated for their cytotoxic activity and potential concentration (CC_{50}) in HepG2 cells and normal hepatocytes. The cells were grown in 96-well plates at a density of 10×10^3 cells/well and incubated at 37ºC in a CO_2 incubator. The cells were treated with various concentrations of each indicated extract (0-4 mg/mL), then incubated overnight. The cell viability rate and cytotoxic concentration were determined using the MTT cell growth test kit (Sigma-Aldrich, Germany), and the amount of formazan dye was assessed by measuring absorbance at 570 nm [19, 20].

Cell morphology and number of survived cells

The variation in cell survival and morphology was achieved by monitoring cell morphology with an inverted microscope and accounting for the number of living cells in response to treatment with *B. pilosa* and *T. portulacastrum* extracts. Cancer cells and normal hepatocytes were planted in a 6-well plate at a density of 10×10^4 cells/well and incubated overnight at 37°C under 5% CO_2 conditions. The cells were then treated with 1 mg/mL of each extract and incubated overnight at the same temperature. The treated cells were monitored using an inverted microscope, and the number of survived cells was manually counted by a hemocytometer [4,5].

Quantitative real-time polymerase chain reaction (qRT-PCR)

The expression of genes was quantified using qRT-PCR, and the cellular total RNA was obtained using TriZol (Invitrogen, USA) and purified using an RNA purification kit (Invitrogen, USA). A M-MLV reverse transcriptase was used to create complementary DNA (cDNA) from 1 μg of total RNA (Promega, USA). The messenger RNA (mRNA) expression of *Raf-1*, MEK1, *LC3B*, and *Atg12* was quantified using the QuantiTect-SYBR-Green PCR Kit (Qiagen, USA). The primers are given in Table 1. In the real-time PCR data analysis, the housekeeping glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA expression level was employed for standardization. The PCR reaction system comprised 10 μL SYBR green, 0.25 μL of RNase inhibitor (25 U/μL), 0.2 M of each primer, 2 μL of synthesised cDNA, and nuclease-free water to a final volume of 25 μL. The PCR conditions were as follows: 95°C for 10 minutes, 40 cycles (95°C for 30 seconds, 60°C for 30 seconds, 72°C for 45 seconds) [21, 22].

Enzyme-linked immunosorbent assay (ELISA)

The released interleukin-1 alpha and beta (IL-1α and IL-1β) were quantified utilizing human ELISA kits (Abcam 46052 and Abcam 46028, respectively). Cells grown in 96-well plates were incubated overnight, then treated with 500 μg/mL of both extracts, followed by an incubation time of (0, 6, 12, 24, 36, 48, and 72 hours).

At each time interval, the cells were lysed using 1× cell lysis buffer (Invitrogen, USA), then 100 µL of the lysed cells were transferred to the ELISA plate reader and incubated for 2 hours at room temperature with 100 µL of control solution and 50 µL of 1× biotinylated antibody. 100 µL of 1× streptavidin-HRP solution was added to each sample and incubated in the dark for 30 minutes, followed by the addition of 100 µL of the chromogen Tetramethylbenzidine (TMB) substrate solution and incubation for 15 minutes at room temperature away from the light, then 100 µL of stop solution was added. At 450 nm, the absorbance of each well was measured [23, 24].

Data analysis

All histograms and final charts were performed by Microsoft Excel. Based on the following equations: Delta-Delta Ct analysis was employed in the quantification analysis of mRNA provided from qRT-PCR assay: (1) Delta-Ct = Ct value for gene- Ct value for GAPDH, (2) (delta–delta Ct) = delta Ct value for experimental –delta Ct for normalized control), (3) Quantification fold change = $(2^{-\text{delta-delta Ct}})$. The significance of all data provided by RT-PCR analysis were statistically analyzed using the student's two-tailed t-test. P-values ≤ 0.05 were regarded statistically significant, whereas p-values ≤ 0.01 were considered highly significant.

Results

Cytotoxic effect and cell viability of HepG2 cells and normal cells upon treatment

The cell viability was interrupted in a dose dependent manner at a concentration of 0.5 mg/mL of *B. pilosa* extract, since the mean absorbance values were decreased upon treatment of *B. pilosa* extract. Moreover, inverted microscope and cell morphology indicated growth inhibition of these cells. On the other hand, *T. portulacastrum* treatment ultimately results in a non-significant difference. Normal hepatocytes treated with both extracts showed non-significant difference in cell morphology when compared to DMSO-treated and non-treated cells (Figure 1A and C). Meanwhile, the cell viability of the normal hepatocytes showed a non-significant toxic effect at the same concentrations of *B. pilosa* treatment (Figure 1B). The CC_{50} of *B. pilosa* extract on the normal cells was almost 1 mg/mL, indicating that the plant agent might disturb the cancer cells at a low concentration with no substantial cytotoxic effect on the normal cells. Furthermore, the number of survived cells was highly significantly down-regulated in HepG2 cells treated with *B. pilosa* extract, whereas the crowdedness of the cells was almost the same, and the cell morphology seemed to be unchanged and revealed a non-significant difference upon treatment with *T. portulacastrum*. (Figure 1D).
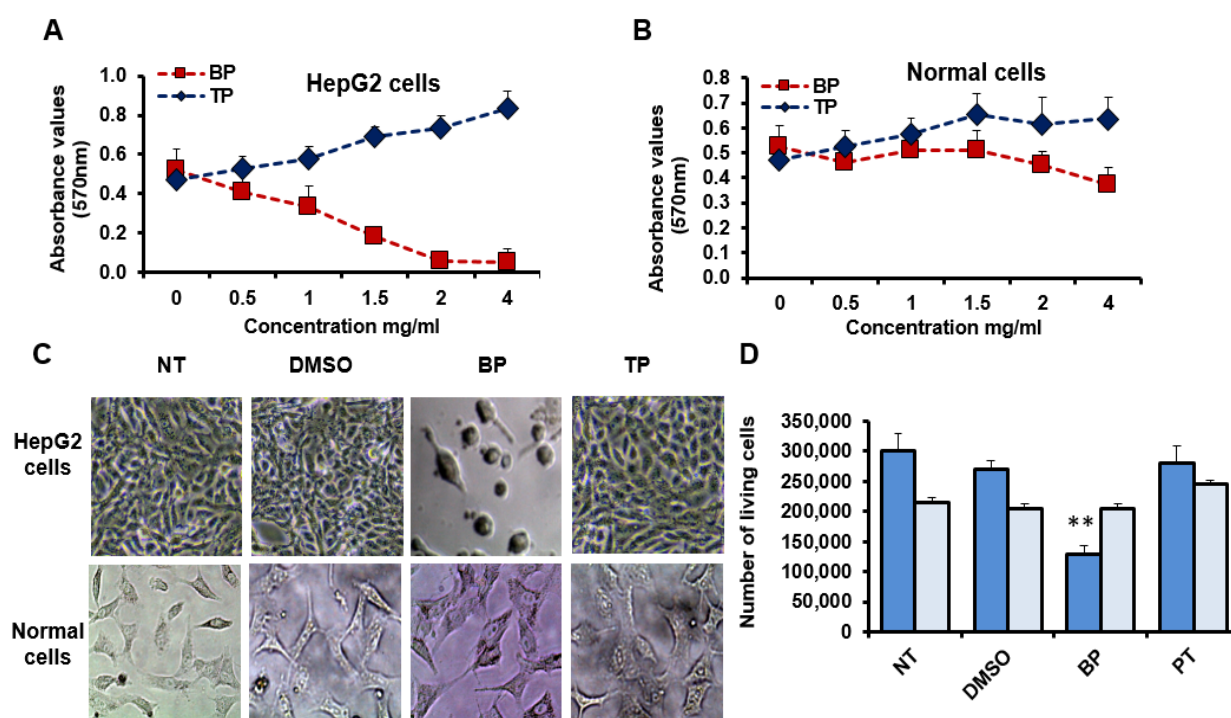

Figure 1. Cytotoxic Effects of Extracted Plants on HepG2 Cells and Normal Hepatocytes. (A) Calculated CC50-dependent cell viability of DMSO extraction of Bidens pilosa and Trianthema portulacastrum on HepG2 cells pre-treated with different concentractions (0-4 mg/mL) of the extracts using MTT assay. (B) Calculated CC50- dependent cell viability of DMSO extraction of Bidens pilosa and Trianthema portulacastrum on normal cells pre-treated with different concentrations (0-4 mg/mL) of the extracts using MTT assay. (C) Representative cell images revealing cell morphology of HepG2 and normal hepatocytes pre-treated with Bidens pilosa and Trianthema portulacastrum extracts. (D) Mean number of survived HepG2 and normal cells upon treatment with DMSO, Bidens pilosa, and Trianthema portulacastrum extracts. Error bars indicate standard devotions (SD) of three independent experiments. SD: Standard deviation. Bp: Bidens pilosa, Tp: Trianthema portulacastrum, NT: Non-treated (control cells), DMSO: Dimethylsulphoxide. **: High significant P ≤ 0.01.

Table 1. Oligonucleotides Sequences Used for mRNA Quantification of Indicated Genes

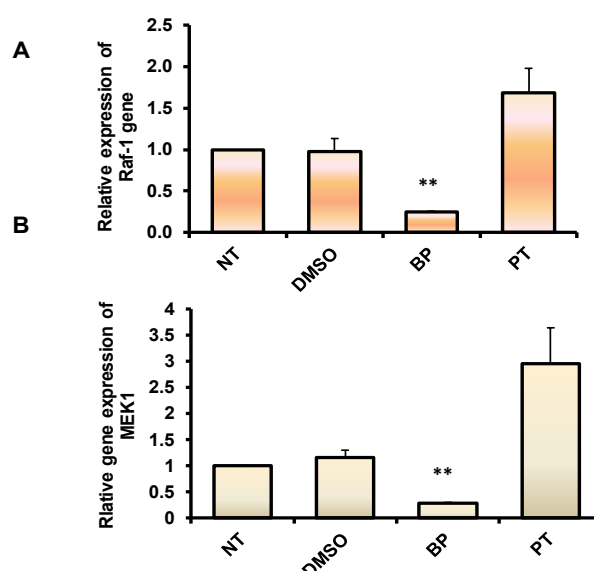| Description	Primer sequences 5'-3'
Raf-1-sense	TTTCCTGGATCATGTTCCCCT
Raf-1 antisense	ACTTTGGTGCTACAGTGCTCA
MEK1-sense	GACCTGCGTGCTAGAACCTC
MEK1-antisense	TCTGGACGCTTGTAGCAGAG
LC3B-sense	AGAGTCGGATTCGCCGCCGCA
LC3B-antisense	GACGGCATGGTGCAGGGATCT
Atg12-senense	CACGAACCATCCAAGGACTCA
Atg12-antisense	TTTGTGGTTCATCCCCACG
GAPDH-sense	TGGCATTGTGGAAGGGCTCA
GAPDH-antisense	TGGATGCAGGGATGATGTTCT

Figure 2. Relative Gene Expression of Cell Proliferation Genes in Treated Cells. (A and B) The relative gene expression of *Raf-1* and *MEK-1* indicated by qRT-PCR in HepG2 cells treated with both extractions and DMSO compared to untreated cells (NT). Error bars indicate SD of three independent experimentAs .Bp: Bidens pilosa, Tp: Trianthema portulacastrum, NT: Non-treated, DMSO: Dimethylsulphoxide, *Raf-1*: RNA activating factor 1, MEK: Mitogen-activated protein kinase. **: High significant at P ≤ 0.01.

Regulation of Raf-1 activation and associated autophagy in HepG2 Cells

Table 2 displays the expression level of *Raf-1*, *MEK-1*, *LC3B*, and *Atg12* genes in the HepG2 cell lines after treatment with DMSO, *B. pilosa*, and *T. portulacastrum* extracts. Relative gene expression of both *Raf-1* and *MEK-1* has been detected in overnight-treated cells using qRT-PCR. The relative expression of *Raf-1*, *MEK-1* genes was highly significantly reduced upon treatment with *B. pilosa* extract, as indicated by the fold change of 0.22 and 0.28 in treated cells compared to DMSO treated and non-treated cells (Figure 2A and B). Also, the expression of *Atg12* and *LC3B* fold changed highly significantly on treating the HepG2 cells with *B. pilosa* DMSO extract (0.27, 0.24) (Figure 3A and B). On the other hand, the treatment with *T. portulacastrum* extract resulted in significantly upregulation of *MEK-1* (2.94) and a non-significant difference in *Raf-1*, *LC3B*, and *Atg12*

(Figure 2 and 3). Collectively, these data revealed that *B. pilosa* extract successfully blocked the Raf/MEK/ERK signaling pathway (the proliferation pathway) and caused a potent inhibition of the autophagy process by regulating autophagy-related genes *LC3B* and *Atg12* as a potential synergistic effect in liver cancer cells. *B. pilosa* extract

Table 2. Quantification Analysis of *Raf-1, MEK1, LC3B* and *Atg12* in HepG2 Cells upon Treatment

Genes	Treatment	GAPDH Mean Ct	Gene Mean Ct	Expression fold changes	Standard deviation	Student two-tails t-test	P-values
Raf-1	NT	19.99	33.85	1	0		
	DMSO	19.67	33.56	0.98	0.15	0.86	> 0.05
	Bp	19.95	33.75	0.22**	0.017	0.002	< 0.01
	Tp	19.35	31.14	1.6	0.294	0.81	> 0.05
MEK1	NT	19.99	31.65	1	0		
	DMSO	19.67	31.06	1.15	0.14	0.26	> 0.05
	Bp	19.95	33.4	0.28**	0.022	0.001	< 0.01
	Tp	19.35	29.14	2.9*	0.69	0.03	> 0.05
LC3B	NT	19.88	29.83	1	0		
	DMSO	20.94	30.66	1.18	0.08	0.09	> 0.05
	Bp	20.95	33.01	0.24**	0.09	0.007	< 0.01
	Tp	19.92	30.1	0.87	0.25	0.53	> 0.05
Atg12	NT	19.88	30.09	1	0		
	DMSO	20.94	30.6	1.51	0.52	0.29	> 0.05
	Bp	20.95	33.14	0.27**	0.13	0.01	< 0.01
	Tp	19.92	30.06	1.08	0.34	0.77	> 0.05

Bp, Bidens pilosa extract; Tp, Trianthema portulacastrum; NT, Non-treated; DMSO, Dimethylsulphoxide; Raf-1, RNA activating .factor 1; *MEK,* Mitogen-activated protein kinase; LC3B, Microtubule-associated proteins 1A/1B light chain 3B; *Atg12*, Autophagy-related 12; *, significant at P ≤0.05; **, high significant at P ≤0.01.
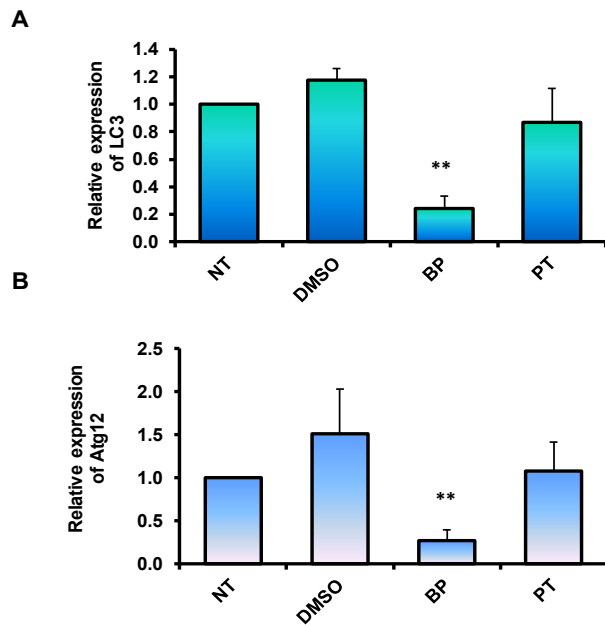
A

B

A

B

Figure 3. Relative Gene Expression of Autophagy-Related Genes in Treated Cells. (A and B) The relative gene expression of LC3 and Atg12 indicated by qRT-PCR in HepG2 cells treated with both extractions and DMSO compared to untreated cells (NT). Error bars indicate SD of three independent experiments .Bp: Bidens pilosa, Tp: Trianthema portulacastrum, NT: Non-treated, DMSO: Dimethylsulphoxide, LC3: Microtubule-associated protein light chain 3, Atg12: Autophagy-related 12. **: High significant at $P \leq 0.01$.

Figure 4. The Concentration of Proinflammatory Cytokines Produced from Treated Cells. (A) The concentration of produced IL-1α (pm/ml) in the fluids media of HepG2 cells subjected to 1mg/mL of Bidens pilosa or Trianthema portulacastrum extracts for the indicated time points compared with untreated cells and DMSO-treated cells. (B) The concentration of produced IL-1β (pm/ml) in pre-treated cells. Bp: Bidens pilosa, Tp: Trianthema portulacastrum, NT: Non-treated, DMSO: Dimethylsulphoxide, IL-1α: Interleukin-1alpha, IL-1β: Interleukin-1 beta, H: Hours.

may serve as a *Raf-1*, *MEK-1*, *LC3B* and *Atg12* inhibitor and apoptotic activator.

Bidens pilosa regulates the secretion of proinflammatory cytokines

As a part of the immune response, inflammation can activate immune cells and induce the production of inflammatory cytokines (IL-1 (α and β) to inhibit the growth of tumors. To further confirm the correlation between activated *Raf-1*, autophagy, and production of proinflammatory cytokines, the transfected HepG2 cells were investigated for their ability to produce (IL-1 (α and β). The ELISA assay showed that the mean concentration of IL-1α and IL-1β was increased to 450 pm/mL and 400 pm/mL respectively in a time-dependent manner for either *Raf-1*, *MEK-1*, *Atg12* or *LC3B* knockdown in *B. pilosa* treated cells, while *T. portulacastrum* DMSO extract reduced the concentration of IL-1α (20 pm/mL) and IL-1β (55 pm/mL) (Figure 4A and B). Collectively, these results showed that downregulation of *Raf-1*, *MEK-1*, *Atg12*, and *LC3B* in *HepG2* cells stimulated the production levels of IL-1 (α and β) and subsequently PCD in transfected cells.

Discussion

Various recent publications demonstrated the effectiveness of many herbal weeds in the treatment of a wide range of diseases [23, 25, 13]. For cancer patients,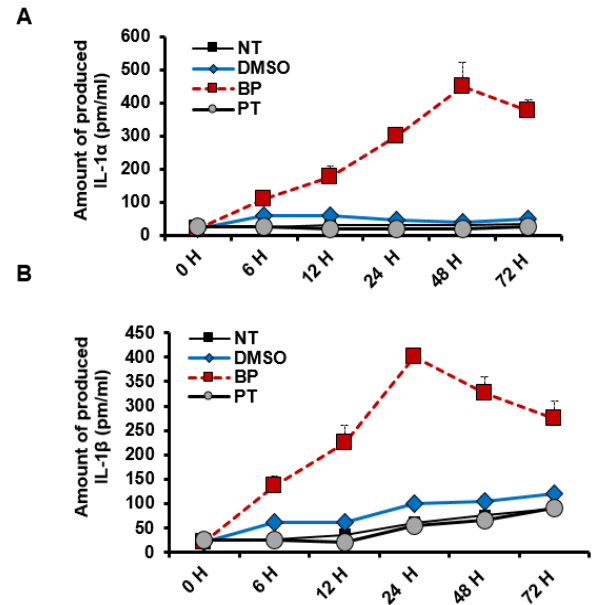 numerous naturally produced herbal formulations are already available. Because most chemotherapy drugs were cytotoxic to normal cells, drug resistance developed. As a consequence, scientific investigation and testing of traditionally used herbs for the treatment of various cancers could be a highly significant source of new chemotherapeutic medications. Weeds are the richest sources of novel pharmaceuticals (Adoniside from Adonis vernalis, Asiaticoside from Centella asiatica, Silymarin from Silybum marianum, etc.) which are effective against many resistant diseases such as cancer and tuberculosis [26]. Numerous studies reported the efficiency of several isolated chemicals from *B. pilosa* leaf extract and proposed that the plant could be used as an anticancer, antioxidant, antimicrobial, and mosquitocidal agent. The whole plant methanolic extract of *B. pilosa* demonstrated a considerable cytotoxic impact against cervical cancer cells (Hela) and a comparable antipyretic activity effect to paracetamol in the rabbit pyrogen test in-vitro. Likewise, the *B. pilosa* petroleum ether leaf extract showed favorable anti-proliferation activity against HepG2 cells [15, 26]. The current study found that the DMSO-leaf extract of *B. pilosa* was cytotoxic to HepG2 cells, with a CC50 of nearly 1 mg/mL on normal cells. HepG2 cell viability was interrupted at a concentration of 0.5 mg/mL upon treatment with *B. pilosa* DMSO extract. While another study found that the CC50 of DMSO extract of *B. pilosa* leaves (North East Indianeco-type) was 0.1 mg/mL [15]. This difference may be due to the difference in the environmental conditions of the study plant. Moreover, many studies examined the anticancer activity of DMSO and others

extracts of different plants against HepG2 cell line. They reported that 0.1 mg/mL silymarin DMSO extract effectively suppressed HepG2 development with minimal deleterious impacts on normal cells [27, 28]. Besides, 50% ethanol-water crude extracts of Diospyros winitii, Terminalia triptera, and Artabotrys harmandii showed cytotoxicity against HepG2 cells with CC_{50} values ranging from 0.1 to 0.5 mg/mL and anticancer activities in-vitro [29, 30]. Aloe vera and Calligonum comosum extracts had cytotoxic activity and were calculated as 0.01 mg/mL for A. vera extract and 0.009 mg/mL for C. comosum extract [31, 32]. The present data confirmed the anticancer activity of *B. pilosa* leaf extracted in DMSO as it highly significant inhibited *Raf-1* and *MEK-1* gene expression. *T. portulacastrum* DMSO extract also inhibited *MEK-1* gene. Another study was quite harmonic to ours, but they used different leaf extract of Hymenosporum flavum and showed promising inhibitory activity against both *Raf-1* and ERK-2 gene expression [33]. *B. pilosa* DMSO extract successfully suppressed the Ras/Raf/MEK/ERK signaling pathway and could regulate the division of HepG2 cells by restoring the sustained Ras/Raf/MEK/ERK signaling pathway and managing programmed cell death and the autophagy process by regulating the autophagy-related genes *LC3B* and *Atg12*. Polygonum cuspidatum was also used in combination with other drugs, in HepG2 and leukemia cell lines, mainly due to its effect on apoptosis. This study agreed with ours which P. cuspidatum extract reduced *LC3B* autophagy-related gene [34]. Moreover, *B. pilosa* extract stimulated P53 as a tumor suppressor gene and its apoptotic signaling pathway and elevated the proinflammatory cytokines IL-1α and IL-1β upon treatment. In agreement with our results, Curcuma Longa, Rabdosia rubescens and Polygonum cuspidatum extracts induced p53 and caused autophagy inactivation of HepG2 cell lines [35].

In conclusion, the current study confirmed the in-vitro activity of *B. pilosa* extract against HepG2 cell proliferation without any detectable cytotoxic effects on the normal hepatic cells. Mechanistically, the anti-proliferation properties of *B. pilosa* extract is due to the blocking of *Raf-1* signaling cascade and autophagosome formation resulting in stimulation of PCD in treated cells. The PCD induced upon *B. pilosa* extract treatment is activated as a result of the increasing level of produced IL-1α and IL-1β. The information presented in this study will be useful in the context of future research and *B. pilosa* DMSO leaf extract could be promising for further preclinical and clinical studies for liver cancer treatment.

Author Contribution Statement

Hany Khalil designed and lead overall the research plan, conceptualized, supervised all experiments. Hany Khalil and Walid Said provided the cell culture experiments and statistical analysis of the quantitative real-time PCR. Sabah Abo-Elmaaty, Abeer Khattab and Saadia Aly assessed in performing the experiments and data analysis. Hany Khalil and Walid Said prepared and wrote the manuscript. All authors have read, edited the manuscript and agreed for publication.

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