Identification of bioactive and anticancer properties of Bidens pilosa L. in-vitro evidence

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Article

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

Posted Date: August 1st, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1875653/v1

Abstract

Hepatocellular carcinoma (HCC) is the most widespread primary liver cancer, with a projected prevalence of more than 1 million cases by 2025. HCC severely affects the entire human population and continues to be a serious global challenge. There is an urgent need for novel therapies to treat and prevent this potentially fatal disease. *Bidens pilosa* and *Trianthema portulacastrum* are noteworthy weeds that include 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 examine the efficiency of *B. pilosa* and *T. portulacastrum* extracts against HCC using the HepG2 cell line. *B. pilosa* and *T. portulacastrum* fresh leaf extracts were prepared using dimethyl sulfoxide. The obtained extracts were evaluated for their cytotoxic activity and potential CC_{50} using the MTT assay in HepG2 cell lines and normal cells. The relative messenger RNA (mRNA) of RAF-1, MEK-1, LC3B, and Atg12 genes was quantified using quantitative reverse-transcription polymerase chain reaction to detect the expression levels of autophagy-related genes in HepG2 cells *in-vitro* for HCC activity. Additionally, an ELISA assay was carried out for the quantification analysis of the released interleukin-1beta (IL-1β) and interleukin-1alpha (IL-1α). *B. pilosa* extract's cytotoxic activity confirmed its potential action on HepG2 cell lines upon treatment compared with 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. The ELISA assay revealed a substantial elevation of the proinflammatory cytokines IL-1α and IL-1β upon treatment. This study found that *B. pilosa* extract had potential inhibitory activities against both RAF-1 and MEK-1 gene expression, and a significant reduction in the relative expression of both LC3B and Atg12 genes upon treatment, without any detectable cytotoxic effects. This extract caused highly significant suppression of the Ras/Raf/MEK/ERK signaling pathway of tumor proliferation and induced an apoptotic signaling pathway. Hence, it could provide a lead structure for drug development strategies against hepatocellular carcinoma to be used in pharmaceutical preparations.

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 hepatologists and interested researchers 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.

Medicinal plants are prospective sources of bioactive phytochemicals, particularly phenolics, that have long been recorded as anticancer agents [3]. *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 [4, 5]. *B. pilosa* has a long history of use in ethnomedicine to treat gastrointestinal and liver ailments, hence it has been recorded as a hepatoprotective and cytotoxic agent against various cancer cells [6]. *T. portulacastrum* is efficacious against heart diseases, inflammation, piles, ascites, alcohol poisoning, and toxin-induced hepatocarcinogenesis [7, 8].

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 [2]. 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, 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 genes and growth factors [9].

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, ageing, liver cancer, and liver ischemia-reperfusion injury [10]. 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 [11, 12, 13].

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° 1056, *Long*. 31°07 50.7) during summer 2020. The collected samples were sterilized with 75% ethanol and ground with liquid nitrogen, then dissolved in 1 mL of DMSO to achieve a final concentration of 100 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 [14, 15]. Inverted microscope (Zeiss A-Plan 10X) was used to image grown cells.

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 cells. 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 [3].

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 [16, 17].

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 g 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). The Raf/MEK/ERK signaling cascades are triggered in response to growth factors from their receptors to particular transcription factors which regulate gene expression and induce apoptotic signaling pathways. The biochemical inhibition of RAF-1 activity could regulate cell proliferation and induce apoptosis. 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) [17, 18].

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 [19, 20].

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

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 control), (3) Quantification fold change = (2-delta-delta Ct) [10, 21]. 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. SDS2.2.2 software was utilized to analyze the qRT-PCR data to drive the Ct values for potential gene expression using delta-delta Ct equations.

Results

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

The cell viability rate of treated HepG2 cells was interrupted at a concentration of 0.5 mg/mL of *B. pilosa* extract. Moreover, inverted microscope 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 **(Fig. 1A& 1C)**. Meanwhile, the cell viability rate of the normal hepatocytes showed a non-significant toxic effect at the same concentrations of *B. pilosa* treatment **(Fig. 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 it revealed a non-significant difference upon treatment with *T. portulacastrum* **(Fig. 1D)**.

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, LC3B, and Atg12 genes was highly significantly reduced upon treatment with *B. pilosa* extract, as indicated by the fold change in treated cells compared to DMSO treated and non-treated cells. On the other hand, *T. portulacastrum* extract resulted in significantly reduced MEK-1 and a non-significant difference in Raf-1, LC3B, and Atg12 **(Fig. 2A, B; Fig. 3A, B)**. Collectively, these data revealed that *B. pilosa* extract successfully blocked the Raf/MEK/ERK signaling 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.

Bidens pilosa **regulates the secretion of proinflammatory cytokines**

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 in *B. pilosa* treated cells, while its concentration was decreased in *T. portulacastrum* treated cells, DMSO treated cells, and untreated cells **(Fig. 4A& 4B)**.

Table 2 Quantification analysis of Raf-1, MEK1, LC3B and Atg12 in HepG2 cells upon treatment

Genes	Treatment	Expression fold changes
Raf-1	NT	1.00
	DMSO	0.98
	BP	0.24**
	TP	1.68
MEK-1	NT	1.00
	DMSO	1.15
	BP	0.28**
	TP	2.94*
LC3B	NT	1.00
	DMSO	1.18
	BP	0.24**
	TP	0.87
Atg12	NT	1.00
	DMSO	1.51
	BP	0.27**
	TP	1.08

BP: *Bidens pilosa* extract, TP: *Trianthema portulacastrum* extract, NT: Non-treated, DMSO: Dimethylsulphoxide, Raf: Rapidly accelerated fibrosarcoma, MEK: Mitogen-activated protein kinase, LC3B: Microtubule-associated proteins 1A/1B light chain 3B, Atg12: Autophagy-related 12.
*: significant at P values ≤ 0.05

**: high significant at P values ≤ 0.01

Discussion

Various recent publications demonstrated the effectiveness of many herbal weeds in the treatment of a wide range of diseases. 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 [22]. The current study found that *B. pilosa* DMSO leaf extract was cytotoxic to HepG2 cells, with a CC_{50} of nearly 1 mg/mL on normal cells. Moreover, many studies examined the anticancer activity of silymarin and reported that 100 µg/mL silymarin DMSO extract effectively suppressed HepG2 development with minimal deleterious impacts on normal cells [23, 24, 25]. 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 100 to 500 µg/mL and anticancer activities *in-vitro* [26]. *Thymus parnassicus* was active against HepG2 cell lines with CC_{50} values of 30 mg/mL [27]. *Aloe vera* and *Calligonum comosum* extracts had cytotoxic activity and were calculated as 10.45 µg/mL for *A. vera* extract and 9.60 µg/mL for *C. comosum* extract [28]. It was reported that by modulating apoptosis, both extracts had anticancer effects against HepG2 cells. 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-vito* [4, 29]. The present data confirmed the anticancer activity of *B. pilosa* DMSO leaf extract as it 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. The green leaves and flowers of guava (*Psidium guajava*) DMSO extract disturbs influenza A virus replication via activation of P53 and its apoptotic-related factors after infection [18]. The leaf extract of *Hymenosporum flavum* showed promising inhibitory activity against both RAF-1 and ERK-2 gene expression [3]. *B. pilosa* extract highly significantly inhibited RAF-1 and MEK-1 gene expression and the autophagy process by regulating the autophagy-related genes LC3B and Atg12. Moreover, it stimulated P53 as a tumor suppressor gene and its apoptotic signaling pathway and elevated the proinflammatory cytokines IL-1α and IL-1β upon treatment.

Conclusion

The current study confirmed the *in-vitro* activity of *B. pilosa* extract against HCC without any detectable cytotoxic effects on the normal hepatic cells. 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.

Abbreviations

HCC: Hepatocellular carcinoma; HepG2: liver cancer cell lines; mRNA: Messenger ribonucleic acid; IL-1α: Interleukin 1 alpha(cytokine); IL-1β: Interleukin 1 beta (cytokine); VACSERA: The Egyptian holding company for biological products and vaccines; BSA: bovine serum albumin; CC50: Cytotoxic concentration of 50%; cDNA: Complementary DNA; qRT-PCR: Quantitative real time polymerase chain reaction; ELISA: Enzyme-linked immunosorbent assay; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; TMB: Tetramethylbenzidine; p53: Tumor protein; Ras: Rat sarcoma; Raf: Rapidly accelerated fibrosarcoma; MEK: Mitogen-activated protein kinase; ERK: Extracellular signal-regulated kinase; *B. pilosa*: *Bidens pilosa* L.; *T. portulacastrum*: *Trianthema portulacastrum* L.; DMSO: Dimethyl sulfoxide; LC3B gene: Microtubule-associated proteins 1A/1B light chain 3B; Atg12 gene: Autophagy related 12 gene; NF-Kb: Nuclear factor kappa-light-chain-enhancer of activated B cells; MCF-7: Human breast cancer cell line; EFM-19: Human breast cancer cell line; MCF-10a: Human breast cancer cell line; TNF-α: Tumor necrosis factor alpha; TGF-β: Transforming growth factor β; IL6: Interleukin 6 (cytokine); IL6: Interleukin 6 (cytokine); HeLa: Human cervical cancer cell line; *A. Vera*: *Aloe vera; C. comosum*: *Calligonum comosum*.

Declarations

Acknowledgements

I would like to begin by thanking my four supervisors: Professors Hani Khalil, Sabah Abo-Elmaaty, Dr. Abeer Khattab, and Dr. Saadia Hamed. It is an often-used cliché, but in this case, it is no overstatement to say that without the consistent guidance, tutelage, support, unparalleled knowledge, and encouragement of my three supervisors, this thesis would never have existed. In particular, I would like to thank Dr. Saadia who went above and beyond to read every line of the manuscript in meticulous detail.

I must say an extremely special thank you to me, suffice it to say, I'm the hero of the whole story.

To my family, particularly my father, the absent present, is always my role model and master, mother and brothers thank you for your love, support, and unwavering belief in me. Without you, I would not be the person I am today.

Above all, I would like to thank my wife for her love and constant support, for all the late nights and early mornings, and for keeping me sane over the past few months. Thank you for being my muse, editor, proofreader, and sounding board. But most of all, thank you for being my best friend. I owe you everything.

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Authors' contributions

Walid Said prepared and wrote the manuscript. Hani Khalil assisted in conceptualize experiments and interpreted data. Walid Said assisted in performing the experiments. Hani Khalil and Walid Said assisted in performing quantitative real-time PCR experiments. Hani Khalil assisted in preparing the graphical abstract. Hani Khalil and Walid Said contributed by performing cell culture experiments and statistical analysis. Hani Khalil designed the research plan, supervised overall research, provided and interpreted data. Walid Said, Sabah A. Abo-Elmaaty, Abeer Khattab and Saadia H. Aly edited and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding

This research received no external funding.

Availability of data and materials

The data that support the results of this study are available from the corresponding author upon reasonable request.

Conflicts of interest

All authors declare that there are no conflicts of interest.

Ethics approval and consent to participate

Plant ethics

B. pilosa erect with yellow flowers with many branched stems, 30:100 cm. Taxonomically, it is dedicated to the *Bidens* genus. *T. portulacastrum* produces colored flowers that are red or white, small, solitary, bisexual, pale pink or white colored. Taxonomically, it is dedicated to the *Trianthema* genus. Plants that were used in this study were collected from Qanatir horticulture research institute fields in Qalyubia, Egypt. (Lat. 30° 1056*, Long*. 31°07 50.7) during the summer of 2020. And were kindly provided by Dr. Abeer A. Khattab, Faculty of Science, Benha University, Egypt. All the procedures of plant collection were conducted to comply with relevant institutional, national and, international guidelines.

Consent for publication

Not applicable

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Figures

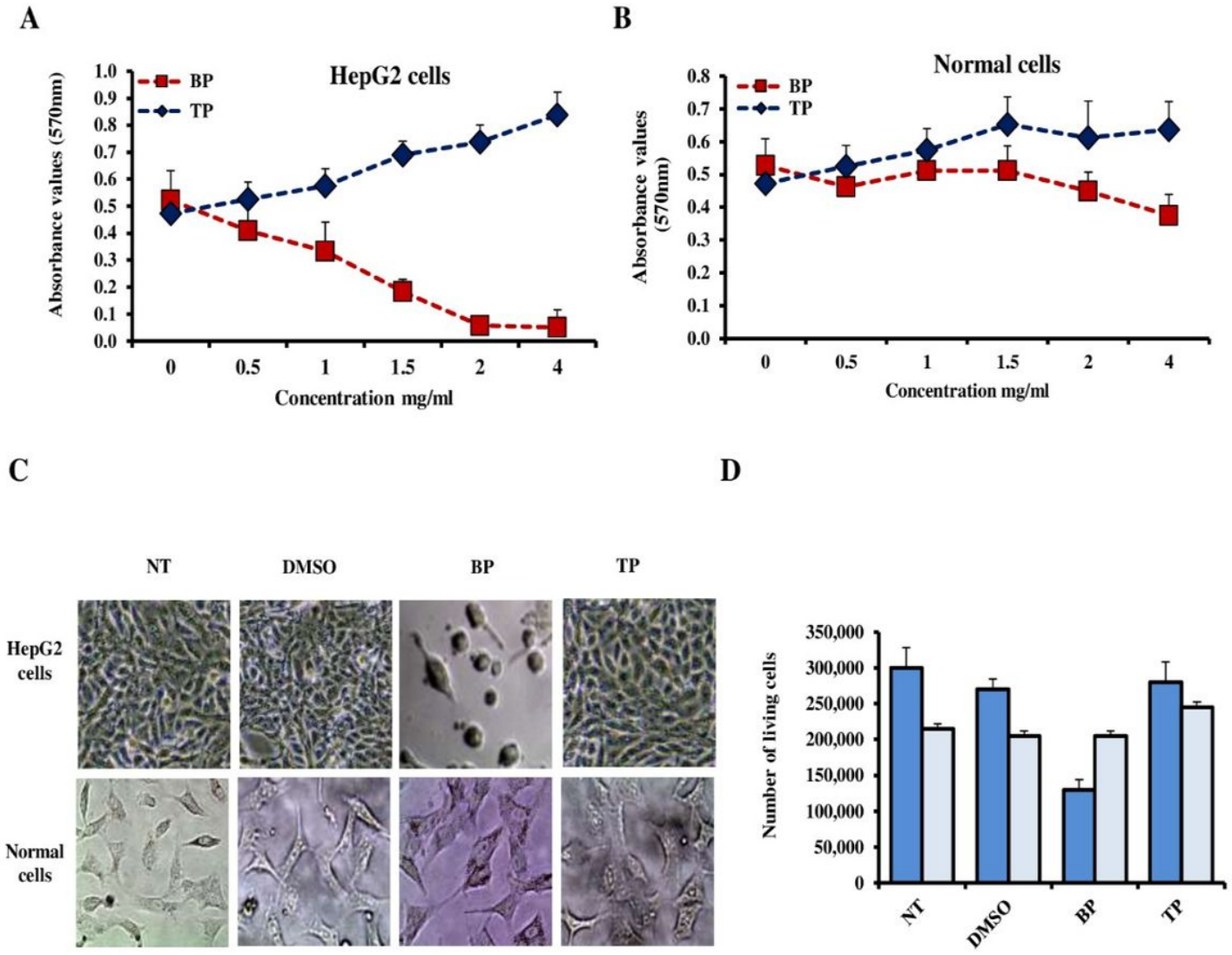

Figure 1

(1A) Calculated CC_{50} -dependent cell viability rate of Bidens pilosa and Trianthema portulacastrum extracts on HepG2 cells pre-treated with different concentractions (0-4 mg/mL) of the extracts using MTT assay. **(1B)** Calculated CC_{50}- dependent cell viability rate of *Bidens pilosa* and *Trianthema portulacastrum* extracts on normal cells pre-treated with different concentractions (0-4 mg/mL) of the extracts using MTT assay. **(1C)** Representative cell images revealing the cell viability of HepG2 and normal hepatocytes cell line pre-treated with *Bidens pilosa* and *Trianthema portulacastrum* extracts. **(1D)** Number of survived cells in HepG2 cells and normal hepatocytes cell upon treatment with DMSO, *Bidens pilosa* and *Trianthema portulacastrum* extracts.

Error bars indicate SD of three independent experiments.

BP: Bidens pilosa, TP: Trianthema portulacastrum, NT: Untreated (control cells), DMSO: Dimethylsulphoxide.

**: Indicates high significant P values ≤0.01

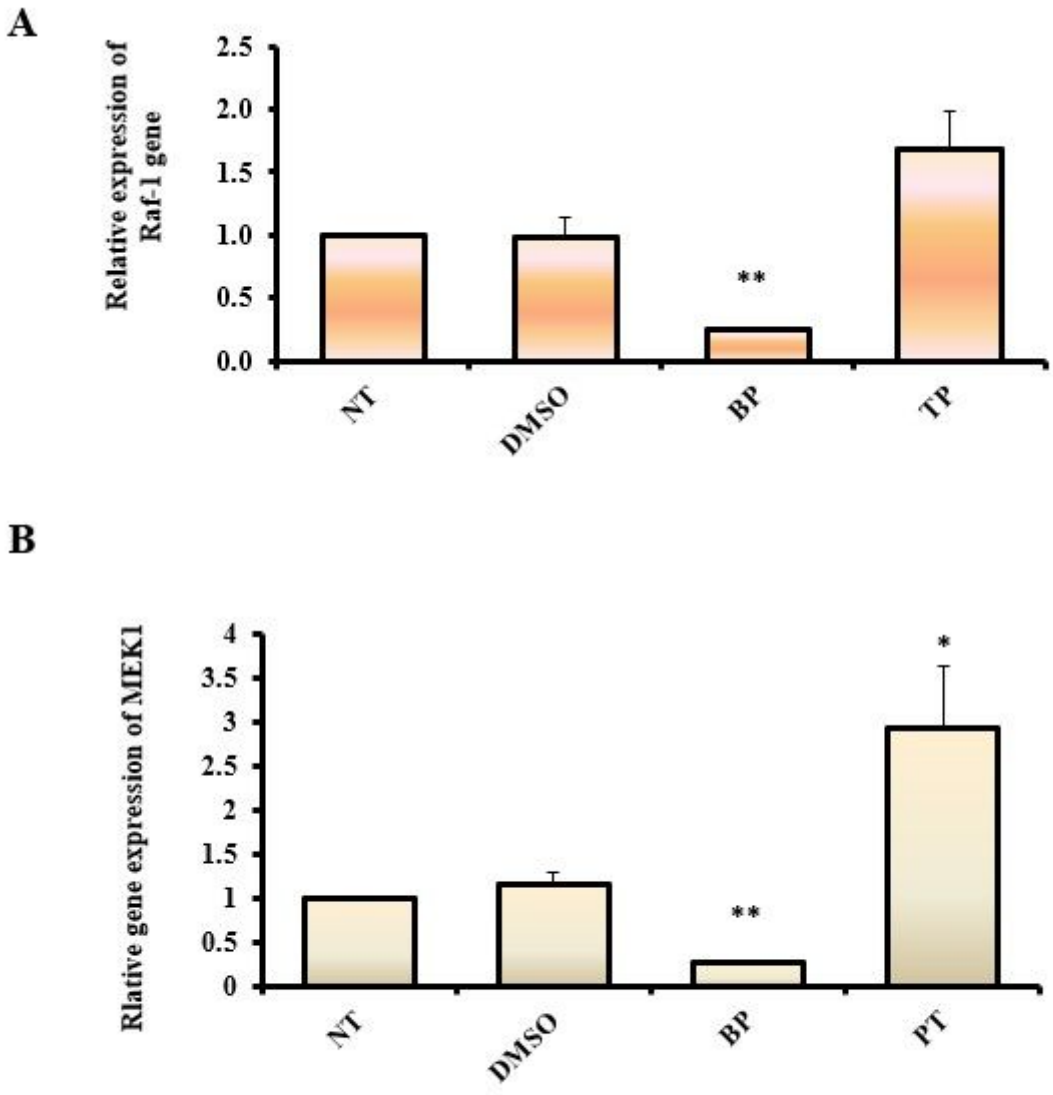

Figure 2

(2A) The relative gene expression of Raf-1 indicated by qRT-PCR in HepG2 cells treated with both extracts and dimethylsulphoxide compared to untreated cells; **(2B)** The relative gene expression of MEKI in treated HepG2 cells in comparison with untreated cells.

Error bars indicate SD of three independent experiments. BP: *Bidens pilosa* extract,TP: *Trianthema portulacastrum* extract, NT: Untreated, DMSO: Dimethylsulphoxide, Raf: Rapidly accelerated fibrosarcoma, MEK: Mitogen-activated protein kinase.

*: significant at P values ≤ 0.05

**: high significant at P values ≤0.01

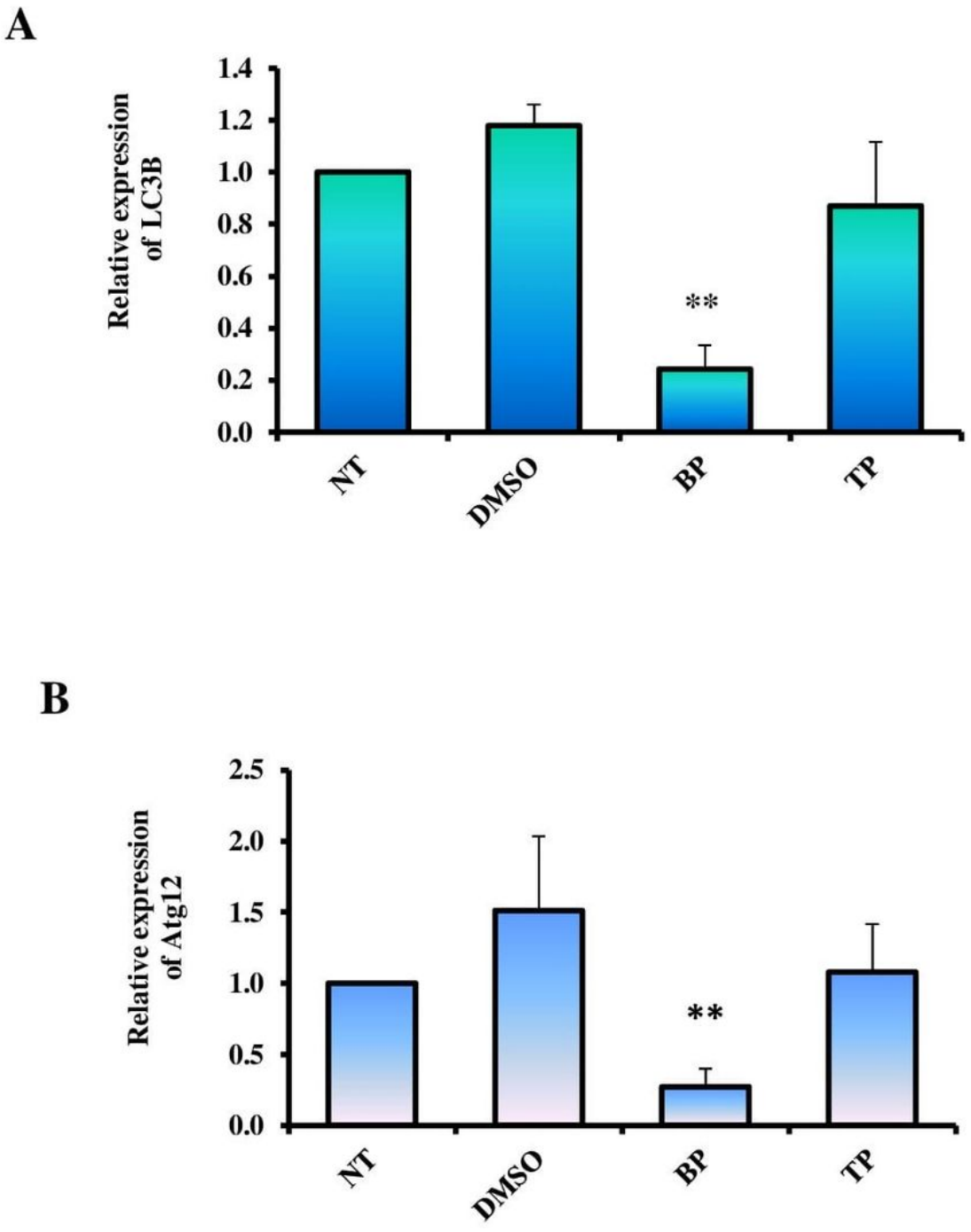

Figure 3

(3A) The relative gene expression of LC3B indicated by qRT-PCR in HepG2 cells treated with 1mg/mL of both extracts and dimethyl sulphoxide compared to untreated cells; **(3B)** The relative gene expression of Atg12 in treated HepG2 cells in comparison with non-treated cells.

Error bars indicate SD of three independent experiments. BP: Bidens pilosa extract, TP: Trianthema portulacastrum extract, NT: Untreated, DMSO: Dimethylsulphoxide, LC3B: Microtubule-associated proteins 1A/1B light chain 3B, Atg12: Autophagy-related 12.

*: Indicates significant at P values ≤ 0.05

**: Indicates high significant atP values ≤0.01

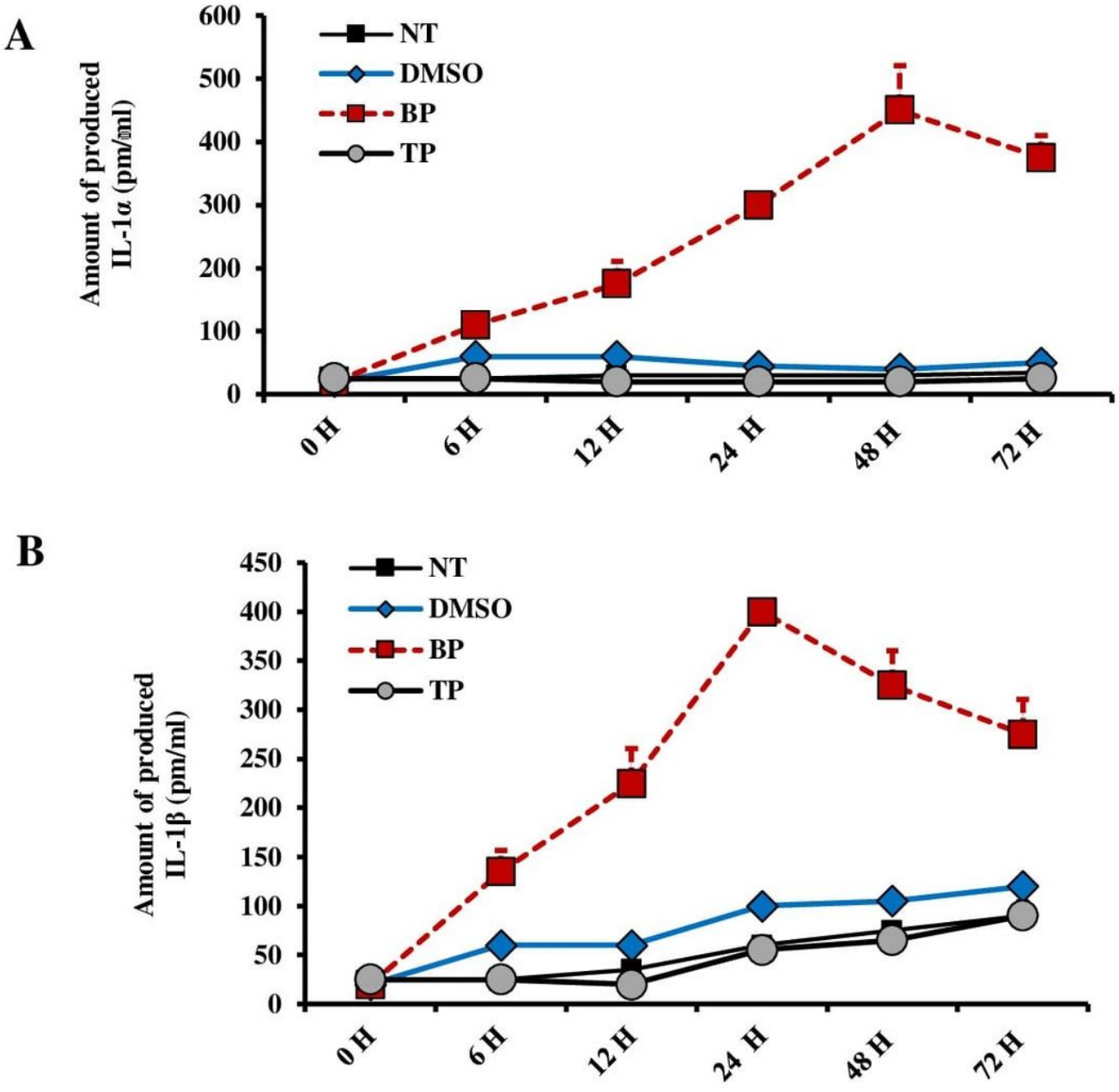

Figure 4

The concentration of proinflammatory cytokines produced in the fluids media of HepG2 cells subjected to 1mg/ml of *Bidens pilosa* and *Trianthema portulacastrum* extracts for the indicated time points compared with untreated cells and DMSO-treated cells. **(4A)** The concentration of produced IL-1α (pm/ml); **(4B)** The concentration of produced IL-1β (pm/ml).

BP: *Bidens pilosa* extract, TP: *Trianthema portulacastrum* extract, NT: Untreated, DMSO: Dimethylsulphoxide, IL-1α:Interleukin- 1α, IL-1β: Interleukin-1β, H: Hour.