Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

Induction of Apoptosis by *Tithonia diversifolia* in Human Hepatoma Cells

Min-Ren Lu[1], Huey-Lan Huang[2], Wen-Fei Chiou[1,3], Ray-Ling Huang[4]

[1]Department of Life Science, National Taitung University, Taitung, [2]Department of Bioscience Technology, College of Health Science, Chang Jung Christian University, Tainan, [3]National Research Institute of Chinese Medicine, Taipei, [4]Department of Biological Science and Technology, Meiho University, Pingtung, Taiwan, ROC

Submitted: 16-05-2016 Revised: 11-07-2016 Published: 13-11-2017

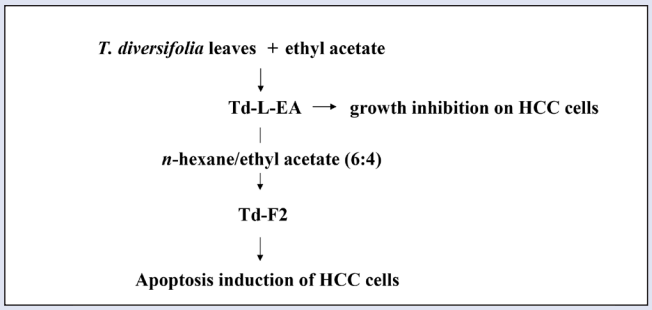
ABSTRACT

Background: Traditional Chinese herb *Tithonia diversifolia*, belonging to the Compositae family, has long been applied for the treatment of liver diseases. In recent years, many reports also indicated that it possesses hepato-protective, anti-inflammatory, and anti-cancer activities. **Objective:** In this study, we evaluated whether *T. diversifolia* is an effective therapy for hepatocellular carcinoma (HCC). **Materials and Methods:** Dry leaves of *T. Diversifolia* were first extracted in ethyl acetate, then further fractionated by different ratio of *n*-hexane-ethyl acetate (8:2→0:1) or methanol as fractions 1-6 (Td-F1 to Td-F6), respectively. We first showed that the ethyl acetate extracts of *T. diversifolia* leaves (Td-L-EA) exhibits growth inhibition on human hepatoma HepG2 cells. To further check the extracts-induced apoptosis, microscopic observation, fragmented chromosomal DNA electrophoresis, apoptotic DNA-detection ELISA assay, flow cytometry, and Western blot analysis were performed. **Results:** After isolating the effective fractions from Td-L-EA, we found strong cytotoxic effects of fraction-2 (Td-F2). By further analyzing the mechanisms of cytotoxic activities using microscopic observation, fragmented chromosomal DNA electrophoresis, apoptotic DNA-detection ELISA assay, and flow cytometry, we found that induction of apoptosis such as DNA fragmentation increased the apoptosis rate and the apoptosis sub-G1 populations in Td-F2-treated HepG2 cells. In addition, we also confirmed Td-F2-induced degradation of caspase-8, caspase-9, caspase-3, and caspase-3 substrate PARP. Besides, Td-F2 also increased the Bcl-2 proapoptotic family protein Bax expression. **Conclusion:** In short, our results clearly showed the induction of apoptosis by ethyl acetate extracts of *T. diversifolia* leaves in human hepatoma HepG2 cells, suggesting its potential application as an antitumor agent.

Key words: apoptosis, caspase, hepatocellular carcinoma, *Tithonia diversifolia*

SUMMARY

- T. *Diversifolia* leaves were first extracted in ethyl acetate, then further fractionated by different ratio of n-hexane/ethyl acetate (8:2→0:1) or methanol.
- These extracts exhibit growth inhibition on human hepatoma (HCC) HepG2 cells.
- *n*-Hexane/ethyl acetate (6:4) extract (Td-F2) induces apoptosis of HCC.



Abbreviations used: *T. diversifolia*, *Tithonia diversifolia*; HCC, Hepatocellular carcinoma DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; HRP, horseradish peroxidase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; OD, optical density; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; PARP, Poly (ADP-ribose) polymerase; PBS, phosphate buffered saline; PI, propidium iodide.

Correspondence:

Dr. Huey-Lan Huang, Department of Bioscience Technology, College of Health Science, Chang Jung Christian University, 1 Changda Rd, Tainan, Taiwan, ROC.
E-mail: hueylanhuang@gmail.com
Dr. Ray-Ling Huang, Department of Biological Science and Technology, Meiho University, Neipu Hsiang, Taiwan, ROC.
E-mail: raylinghuang2093@gmail.com
DOI: 10.4103/0973-1296.218113

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Tithonia diversifolia (*T. diversifolia)* is a kind of flowering plants in *Compositae* family. Also known as Tithonia or Mexican sunflower, it is native to eastern Mexico and Central America, and is traditionally used as a Chinese herbal medicine for chronic hepatitis, liver diseases, detoxification, diarrheas, wound healing, and fever relief.[1]

In the last two decades, pharmacologic or medical plant researches have shown more functions of *T. diversifolia*, including potential treatment for cancers, hyperglycemia, diabetes, inflammation, and malarial and gastric ulcers.[1-11] Some components of *T. diversifolia* are possibly involved in the treatment of these diseases: chlorogenic acids are correlated with anti-inflammation[4] or neutrophil locomotion;[5] sesquiterpenes might be useful for hyperglycemia and diabetes partially due to induction of peroxisome proliferation;[1,3] and flavonoids and sesquiterpenes such as tagitinin C are correlated with antiproliferative activity against leukemia, colon cancer, glioblastoma, and hepatoma.[2,6-10]

Apoptosis refers to programmed cell death that has been studied for a while. There are many well-known molecules involved in apoptosis decision, such as caspases, and proapoptotic or anti-apoptotic Bcl-2 family members.[12,13] The unique features of apoptosis include DNA fragmentation. Many well-known chemotherapeutic agents like irradiation or cisplatin trigger apoptosis of cancer cells.[14] Recently, there have been many findings about other types of programmed cell death, autophagic[6,7,15,16] or necrotic cell death, in the absence of DNA fragmentation in nuclei.[13,17-19]

In this study, we focused on the ethyl acetate extracts of *T. diversifolia* leaves (Td-L-EA), which exhibit cytotoxicity in human hepatoma

Cite this article as: Lu MR, Huang HL, Chiou WF, Huang RL. Induction of apoptosis by *Tithonia diversifolia* in human hepatoma cells. Phcog Mag 2017;13:702-6.

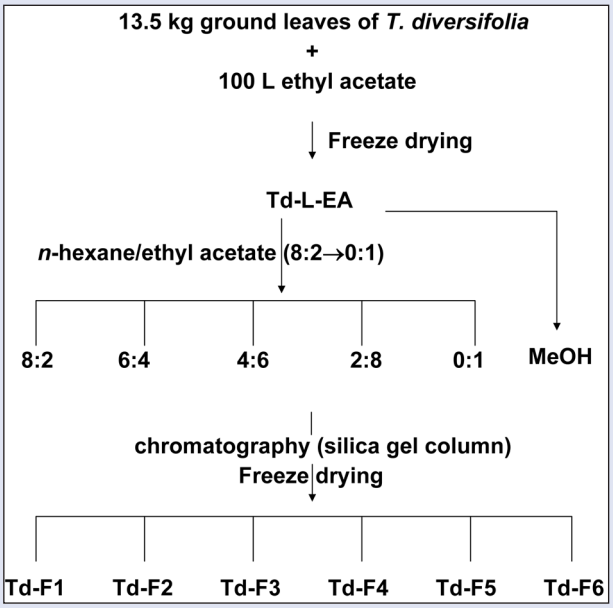


Figure 1: Preparation of *T. diversifolia* extracts. Dry leaves of *T. diversifolia* were first extracted with ethyl acetate as Td-L-EA fraction after subjecting to freeze-drying process. To fractionate, Td-L-EA powder was then dissolved in *n*-hexane-ethyl acetate (8:2→0:1) or methanol (Td-F1 to Td-F6) and chromatographed as described in "Materials and Methods" section.

HepG2 cells. After further fractionation, we found fraction 2 (Td-F2) causes cytotoxicity and apoptosis of HepG2 cells according to DNA gel electrophoresis, ELISA kit, and immunoblotting.

MATERIALS AND METHODS

Cell culture

Human hepatoma HepG2 cells and Chang liver cells were cultured in DMEM (Gibco) with 10% (v/v) fetal calf serum (Hyclone), 1% penicillin-streptomycin (Gibco), 1% nonessential amino acid (Gibco), 0.1% fungizone (Sigma), and 2 mM l-glutamine (Sigma). Cells were then cultured in a humidified atmosphere of 5% CO_2 at 37°C. For cell treatment, freeze-dried fractions were dissolved in dimethyl sulfoxide (DMSO, Sigma) as 400-fold stocks. Camptothecin (Sigma, purity ≥ 95%) or podophyllotoxin (Sigma, purity ≥ 98%) treatment was used as the positive control in comparison with the tested extracts for the following assays.

Extraction of *T. diversifolia* and fractionation

T. diversifolia was kindly identified and collected by Dr. Hung-Liang Lai (Department of Plant Industry, National Pingtung University of Science and Technology) from his institute in September 2009. As shown in Figure 1, dry leaves of *T. diversifolia* (13.5 kg) was first ground and soaked in 1 L ethyl acetate for 3 days. This procedure was repeated eight times. After subjecting to freeze-drying process, we obtained the Td-L-EA fraction, which was then dissolved in *n*-hexane-ethyl acetate (8:2→0:1) or methanol, and chromatographed by a silica gel column [Figure 1]. The first to sixth fractions are denoted as Td-F1 to Td-F6, respectively.

MTT assay

We used MTT assay to validate extracts-induced growth inhibition or cytotoxicity. The assay was done in triplicate. Overnight-cultured 2 × 105 per mL cells in a 96-well plate were incubated in the presence or absence of different concentrations of extracts for 24 or 48 h, then cells were incubated with 20% 5-mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) at 37°C for another 4 h. After culture medium removal, 100 μL DMSO was used to dissolve the dark blue crystals inside cells, and the optical density (OD) values of the plates were measured at 540 nm by a Power Wavex Microelisa reader (Bio-tek). Growth inhibition was calculated as follows: mean OD values of untreated control cells were designated as 100% viability.

Cell cycle assessment by flow cytometry

After Td-F2 extracts treatment, cells were trypsinized, collected, and resuspended in ethanol for fixation. Cells were then washed with PBS buffer, suspended with PBS buffer containing 0.1 mg/mL RNase A and 0.5% Tritone X-100 for 1 h, mixed with propidium iodide (PI) (20 μg/mL).[18] The DNA content inside the cells was measured and analyzed by flow cytometry (FACS can, Becton Dickenson).[16]

Detection of fragmented chromosomes inside apoptotic cells by agarose gel electrophoresis

After Td-F2 extracts treatment for 24 h, cells from 10-cm dish were trypsinized, collected, and resuspended in 400 μL extraction buffer (50 mM Tris–HCl pH 8.0, 10 mM EDTA pH 8.0, 0.3% Triton X-100) on ice for 30 min. The reaction was then mixed with 10 μL RNase A (20 mg/mL, BBI) at 55°C for 30 mins and mixed with 8 μL proteinase K (20 mg/mL, Focus) at 55°C for another 30 min. Finally, chromosomal DNA inside the reaction was purified with phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with ethanol and NaOAc, resuspended, and detected by 1.5% agarose gel electrophoresis.

Apoptotic DNA-detection ELISA assay

After treatment with extracts for 24 h, cells with apoptotic DNA (cytosolic histone-bound DNA fragments) were analyzed using the Cell Death Detection ELISAPLUS Kit (Roche Biochemicals) as described by the manufacturer. Briefly, the culture medium was removed carefully by aspiration and 200 μL lysis buffer was added and incubated with shaking for 30 min at room temperature. After removal of debris by centrifugation, cell lysates were placed into a microtiter plate coated with streptavidin and incubated with anti-histone-biotin and anti-DNA conjugate to horseradish peroxidase (HRP) at room temperature for 2 h. The wells were then washed three times to remove unbound reagents and quantities of nucleosomes were determined by the retained peroxidase using 2,2'-azino-di[3-ethylbenzthiozolin-sulfonate] as substrate and by the absorbance of OD 405 nm.

Western blot analysis

After Td-F2 extracts treatment for 24 h, cells were collected and dissolved in Mammalian Protein Extraction Buffer (GE Healthcare) with protease inhibitors (Roche Biochemicals). After debris removal and protein quantitation, cell lysates were then subjected to 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with the following antibodies: caspase-8, caspase-9, caspase-3, PARP (Cell Signaling), Bax, Bcl-xL, and actin (Santa Cruz Biochemicals).

RESULTS

Since *T. diversifolia* is traditionally used for treating chronic hepatitis and liver diseases in Chinese herbal medicine, our goal is to clarify whether *T. diversifolia* is in fact effective in hepatoma treatment. As compared with buds or branches, we found that the ethyl acetate extract from leaves of *T. diversifolia* (i.e., Td-L-EA) exhibits much stronger inhibition

Table 1: Growth inhibition of human HCC cells by *T. diversifolia* extracts.

treatment		% survival								
h	(μg/ml)	HepG2 cells							Chang liver cells	HepG2 cells
		Td-L-EA	Td-F1	Td-F2	Td-F3	Td-F4	Td-F5	Td-F6	Td-F2	Cam 10 μM
48	1.56			78.45 ± 5.90					94.50 ± 3.52	15.25 ± 2.08***
	3.13	103.42 ± 3.99	101.44 ± 2.30	39.43 ± 3.59***	94.69 ± 2.04	95.76 ± 2.43	95.03 ± 1.83	98.19 ± 3.13	83.81 ± 1.05	
	6.25	82.13 ± 8.69	104.31 ± 0.70	25.52 ± 2.84***	61.90 ± 4.17***	99.45 ± 0.76	96.18 ± 1.19	98.55 ± 1.32	60.51 ± 3.26**	
	12.5	52.11 ± 0.77***	102.82 ± 0.86	19.78 ± 0.54***	22.98 ± 0.50***	86.97 ± 7.83	91.63 ± 1.59	96.05 ± 2.82	51.03 ± 2.43***	
	25	23.73 ± 0.74***	96.63 ± 2.54	14.41 ± 1.66***	18.15 ± 0.28***	65.53 ± 4.71**	90.81 ± 2.55	99.63 ± 1.50	17.66 ± 8.47***	
	50	19.11 ± 2.11***	77.30 ± 1.71***	2.72 ± 0.34***	17.74 ± 0.61***	31.96 ± 1.51***	94.47 ± 3.03	93.89 ± 6.01	2.68 ± 0.36***	
	100	7.22 ± 0.28***	23.27 ± 2.02***	1.71 ± 0.36***	2.28 ± 0.31***	5.34 ± 1.27***	11.15 ± 1.00***	16.97 ± 3.48***	2.29 ± 0.17***	
24	1.56			97.69 ± 1.67					112.98 ± 6.26	
	3.13			67.34 ± 3.3					107.70 ± 7.36	
	6.25			50.71 ± 0.95***					102.58 ± 2.49	

HepG2 HCC cells or Chang live cells as indicated were left untreated, treated with Td-F1 to Td-F6 extracts or 10 μM camptothecin (positive control) for 48 h, and the viability of cells was determined by MTT assay. Mean OD values of untreated control were calculated as 100% viability. Each value represents the mean ± SEM obtained from three repeats. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ (*t*-test) as compared with untreated control

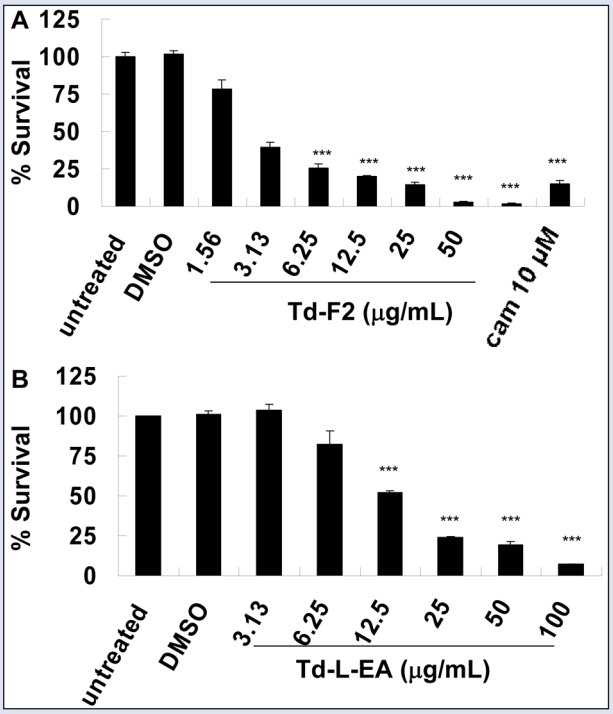


Figure 2: Cytotoxic activity of Td-F2 extracts in human hepatoma cells. HepG2 hepatoma cells were left untreated, treated with 0.25% DMSO (solvent control), various concentrations of Td-F2 (A) or Td-L-EA extracts (B) as indicated or 10 μM camptothecin (positive control) for 48 h, and the viability of cells was determined by MTT assay. Mean OD values of untreated control were calculated as 100% viability. ***$P < 0.001$ (*t*-test) as compared to untreated control

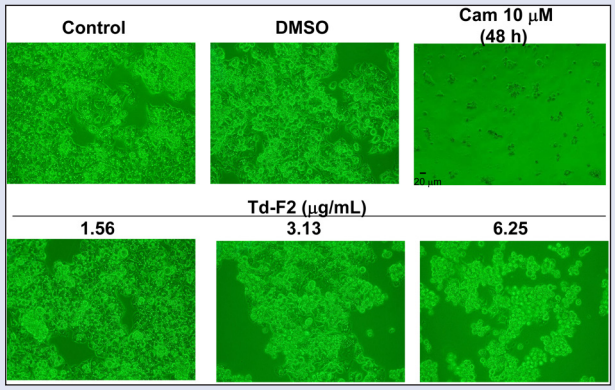
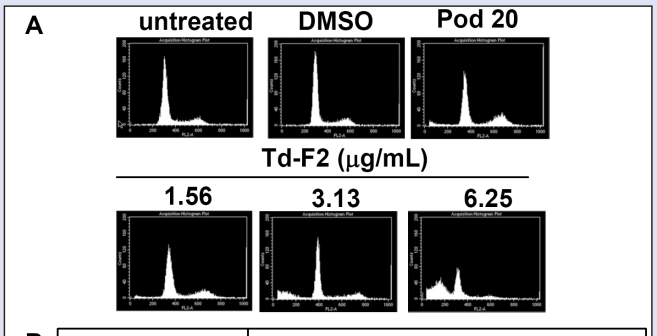


Figure 3: Morphology of Td-F2 extracts-treated human hepatoma cells. HepG2 hepatoma cells were left untreated, treated with 0.25% DMSO (solvent control), various concentrations of Td-F2 extracts as indicated for 24 h or 10 μM camptothecin (positive control) for 48 h. Cell morphology was observed by microscopy

on human hepatoma cells HepG2 proliferation activities (data not shown), so we focused on cytotoxic activities of Td-L-EA in this study. In order to trace the active components, we next fractionated Td-L-EA with methanol (Td-F6) or *n*-hexane and ethyl acetate mixture (8:2, 6:4, 4:6, 2:8, or 0:1 for Td-F1 to Td-F5, respectively) as shown in Figure 1 and "Material and Methods" section. We first tested the growth inhibition of HepG2 hepatoma cells by these extracts. Our results show that after 48-h treatment and MTT assay, IC_{50} values of Td-L-EA and Td-F1 to Td-F6 are 13.5, 70.54, 3, 7.97, 34.11, 61.19, and 77.14 μg/mL, respectively, consistent with the original ethyl acetate extracts (Td-L-EA). From these results, we can see that the two most effective extracts in terms of growth inhibition in HepG2 HCC cells are those after further extraction of Td-L-EA with *n*-hexane and ethyl acetate mixture (i.e., 6:4 and 4:6 for Td-F2 and Td-F3, respectively) [Table 1 and Figure 1 and Figure 2]. SinceTd-F2 extract is also less toxic to normal hepatocytes, Chang liver cells [Table 1], we focus on Td-F2 extracts in this study.

To gain insights into the growth inhibition in hepatoma cells of Td-F2 extracts, we next investigated whether Td-F2 extracts induce cytotoxicity or cell cycle arrest in HepG2 hepatoma cells. As shown in Figure 3, Td-F2 extracts cause cell death or growth arrest according to morphology observation. We then checked if the cytotoxic effect of Td-F2 extracts is due to apoptosis. As shown in Figure 4, Td-F2 extracts increase G0/G1 cell cycle population. In addition, 6.25 μg/mL Td-F2 extracts cause 50% sub-G1 cells (cells with fragmented chromatin)[18] by flow cytometry, suggesting apoptosis induction by Td-F2 extracts in HepG2



B

		(%)			
		Sub-G1	G0/G1	S	G2/M
untreated		0.72	73.85	18.15	8
DMSO		0.19	76.46	15.76	7.78
Td-F2 (μg/mL)	1.56	1.11	70.29	15.36	14.35
	3.13	15.48	76.03	15.26	8.71
	6.25	50.67	83.86	10.75	5.89
Pod (20 μg/mL)		3.24	64.16	11.10	24.74
Cam (10 μM)		31.5	79.4	11.4	9.2

Figure 4: Induction of fragmented chromosomal DNA by Td-F2 extracts in hepatoma cells. HepG2 hepatoma cells were left untreated, treated with DMSO (solvent control), various concentrations of Td-F2 extracts as indicated, positive control podophyllotoxin 20 μg/mL (Pod) or 10 μM camptothecin (Cam) for 24 h. Cells were trypsinized, fixed, and stained with PI. Cell cycle distribution was analyzed by flow cytometry as described in "Material and Methods" section. Percent G0/G1 cells include cells with 2N chromosomal DNA and with DNA less than 2N (sub-G1)

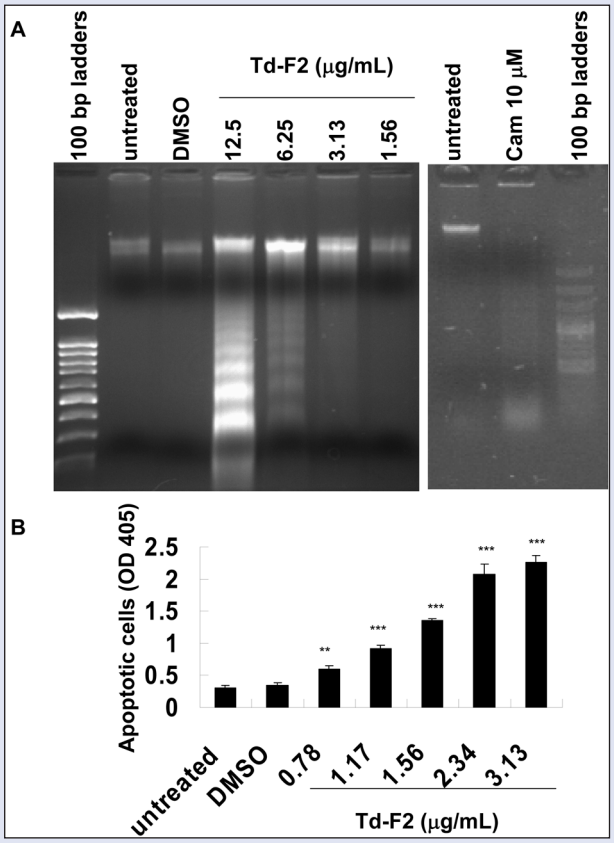


Figure 5: Induction of apoptosis by Td-F2 extracts in hepatoma cells. HepG2 cells were left untreated, treated with DMSO, various concentrations of Td-F2 extracts, or positive control 10 μM camptothecin (Cam) podophyllotoxin 20 μg/mL (Pod) for 24 h. After cells were lyzed, chromosomal DNA inside cells was purified and analyzed by agarose electrophoresis (A) or cells with apoptotic DNA (cytosolic histone-bound DNA fragments) were analyzed by the Cell Death Detection ELISAPLUS Kit (B). $^{**}P < 0.01$ and $^{***}P < 0.001$ (*t*-test) as compared with untreated control

hepatoma cells. Finally, we confirmed Td-F2 extracts-induced apoptosis by detection of fragmented chromosomal DNA using agarose gel electrophoresis [Figure 5A] and apoptotic DNA-detection ELISA assay [Figure 5B]. These data indicate Td-F2 extracts indeed cause apoptosis of HepG2 hepatoma cells.

To further explore Td-F2 extracts-induced apoptosis, we checked the involvement of caspases and other apoptotic proteins by immunoblotting. As shown in Figure 6, degradation of procaspase-3, -8, and -9 is detected in Td-F2-treated cells, indicating the activation of apoptotic key enzymes. Td-F2 extracts also cause degradation of poly (ADP-ribose) polymerase (PARP), one of caspase-3 substrates and the cutting of the function of the DNA repair enzyme. In addition, we found an increase of proapoptotic protein Bax and no obvious change of anti-apoptotic protein Bcl-xL after treatment of Td-F2 extracts for 24 h. According to [Figure 6], Bcl-xL is probably not involved, or is involved in Td-F2 extracts-induced apoptosis at other time points. These data suggest the involvement of intracellular levels of apoptosis markers caspase-3, -8, -9, PARP, and Bax in Td-F2 extracts-induced apoptosis.

DISCUSSION

T. diversifolia is used in treatment of chronic hepatitis and liver diseases in Chinese herbal medicine. In this study, we further demonstrated the potential treatment of hepatoma by *T. diversifolia* extracts. We found cytotoxicity induced by ethyl acetate extracts of *T. diversifolia* leaves (Td-L-EA) in human HepG2 hepatoma cells [Table 1], consistent with ethnopharmocologic use of *T. diversifolia*. Further fractionation of *T. diversifolia* extracts by methanol (Td-F6) or *n*-hexane and ethyl acetate mixture (8:2, 6:4, 4:6, 2:8, or 0:1 for Td-F1 to Td-F5, respectively) is demonstrated in Figure 1. As compared with the original Td-L-EA extracts, the cytotoxic components for HepG2 hepatoma cells are concentrated in *n*-hexane and ethyl acetate (6:4) mixture-extracted fraction 2 (Td-F2) [Table 1 and Figure 2] due to the strongest cytotoxicity.

To elucidate the cytotoxic mechanisms of *T. diversifolia* leaves (Td-L-EA), we have shown Td-F2-induced apoptosis by microscopic observation [Figure 3], fragmented chromosomal DNA electrophoresis, and apoptotic DNA-detection ELISA assay [Figure 5]. In addition, we have also shown that 6.25 μg/mL Td-F2 extracts cause 50% sub-G1 cells and G0/G1 cell cycle arrest by flow cytometry in HepG2 hepatoma cells [Figure 4]. Moreover, we have also found Td-F2 extracts-induced degradation of caspase-8, -9, and -3 and activation of caspase-3 through the detection of caspase-3 substrate PARP degradation [Figure 6]. Finally, Td-F2 extracts also increase proapoptotic Bcl-2 family member Bax [Figure 6]. In fact, not only for HepG2 cells, we have also found Td-F2-induced apoptosis of Hep3B hepatoma cells by flow cytometry, fragmented chromosomal DNA electrophoresis, and apoptotic DNA-detection ELISA as well (data not shown). In short, all these results suggest apoptosis induction by Td-F2 extracts in human HepG2 and Hep3B hepatoma cells through caspase-8, -9, -3, and Bax-involved pathways, similar to many extracts or components from herbs used in Chinese medicine.[20,21]

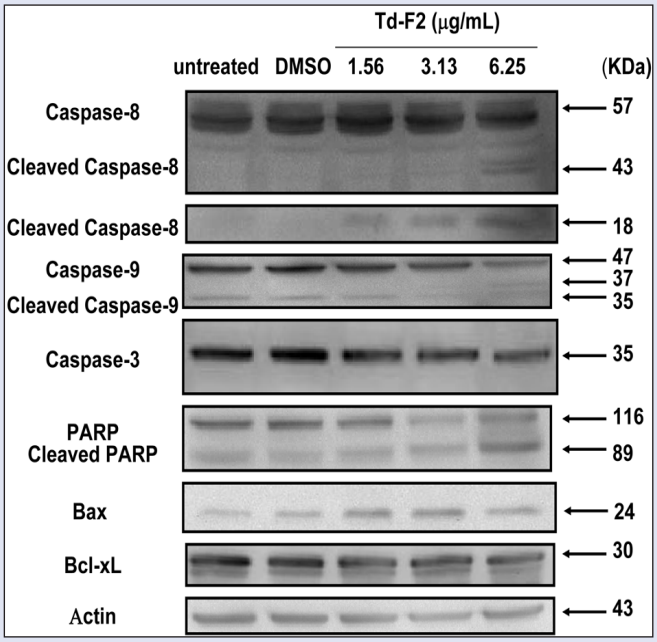


Figure 6: Involvement of apoptosis-related proteins in Td-F2-induced apoptosis in human hepatoma cells. After treatment with DMSO or various concentrations of Td-F2 extracts for 24 h, HepG2 cell lysates were collected and subjected to 12.5% SDS-PAGE. Immunoblotting was done using antibodies against caspase-8, -9, -3, PARP, Bax, Bcl-xL, and actin as indicated

Our findings are consistent with previous studies about induction of cytotoxic activity against hepatoma, leukemia, glioblastoma, or colon cancer cells by *T. diversifolia* components, such as some sesquiterpenes.[2,6,9,10] However, the main difference between ours and their study is the use of ethyl acetate instead of methanolic or EtOH extraction for the first step.[2,6,9,10] Tagitinin C from methanol extracts of *T. diversifolia* also induces apoptosis of hepatoma cells but induces autophagic cell death of glioblastoma cells.[6-8] Finally, the cytotoxic mechanisms of other sesquiterpenes need to be further studied.[2,9]

In conclusion, our findings indicate the exhibition of cytotoxicity and induction of apoptosis by Td-F2 extracts from *T. diversifolia* in human hepatoma cells. We will further study the active components correlated with induction of apoptosis in our Td-F2 extracts and report the findings in the future.

Acknowledgements

This work was supported in part by Grants from the National Science Council, Taiwan (NSC 93-2320-B-276-001), and ROC Ministry of Science and Technology (MOST 103-2313-B-309-001, MOST 104-2320-B-309-001 and MOST 105-2320-B-309-002).

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Zhao G, Li X, Chen W, Xi Z, Sun L. Three new sesquiterpenes from *Tithonia diversifolia* and their anti-hyperglycemic activity. Fitoterapia 2012;83:1590-7.
2. Kuroda M, Yokosuka A, Kobayashi R, Jitsuno M, Kando H, Nosaka K, *et al.* Sesquiterpenoids and flavonoids from the aerial parts of *Tithonia diversifolia* and their cytotoxic activity. Chem Pharm Bull (Tokyo) 2007;55:1240-4.
3. Lin HR. Sesquiterpene lactones from *Tithonia diversifolia* act as peroxisome proliferator-activated receptor agonists. Bioorg Med Chem Lett 2012;22:2954-8.
4. Chagas-Paula DA, Oliveira RB, da Silva VC, Gobbo-Neto L, Gasparoto TH, Campanelli AP, *et al.* Chlorogenic acids from *Tithonia diversifolia* demonstrate better anti-inflammatory effect than indomethacin and its sesquiterpene lactones. J Ethnopharmacol 2011;136:355-62.
5. Hebeda CB, Bolonheis SM, Nakasato A, Belinati K, Souza PD, Gouvea DR, *et al.* Effects of chlorogenic acid on neutrophil locomotion functions in response to inflammatory stimulus. J Ethnopharmacol 2011;135:261-9.
6. Lee MY, Liao MH, Tsai YN, Chiu KH, Wen HC. Identification and anti-human glioblastoma activity of tagitinin C from *Tithonia diversifolia* methanolic extract. J Agric Food Chem 2011;59:2347-55.
7. Liao MH, Lin WC, Wen HC, Pu HF. *Tithonia diversifolia* and its main active component tagitinin C induce survivin inhibition and G2/M arrest in human malignant glioblastoma cells. Fitoterapia 2011;82:331-41.
8. Liao MH, Tsai YN, Yang CY, Juang CL, Lee MY, Chang LH, *et al.* Anti-human hepatoma Hep-G2 proliferative, apoptotic, and antimutagenic activity of tagitinin C from *Tithonia diversifolia* leaves. J Nat Med 2013;67:98-106.
9. Gu JQ, Gills JJ, Park EJ, Mata-Greenwood E, Hawthorne ME, Axelrod F, *et al.* Sesquiterpenoids from *Tithonia diversifolia* with potential cancer chemopreventive activity. J Nat Prod 2002;65:532-6.
10. Kuo YH, Chen CH. Sesquiterpenes from the leaves of *Tithonia diversifolia*. J Nat Prod 1998;61:827-8.
11. Sanchez-Mendoza ME, Reyes-Ramirez A, Cruz Antonio L, Martinez Jimenez L, Rodriguez-Silverio J, Arrieta J. Bioassay-guided isolation of an anti-ulcer compound, tagitinin C, from *Tithonia diversifolia*: role of nitric oxide, prostaglandins and sulfhydryls. Molecules 2011;16:665-74.
12. Mak TW, Yeh WC. Signaling for survival and apoptosis in the immune system. Arthritis Res 2002;4 (Suppl 3): S243-52.
13. Galluzzi L, Aaronson SA, Abrams J, Alnemri ES, Andrews DW, Baehrecke EH, *et al.* Guidelines for the use and interpretation of assays for monitoring cell death in higher eukaryotes. Cell Death Differ 2009;16:1093-107.
14. Huang HL, Fang LW, Lu SP, Chou CK, Luh TY, Lai MZ. DNA-damaging reagents induce apoptosis through reactive oxygen species-dependent Fas aggregation. Oncogene 2003;22:8168-77.
15. Chen YJ, Chi CW, Su WC, Huang HL. Lapatinib induces autophagic cell death and inhibits growth of human hepatocellular carcinoma. Oncotarget 2014;5:4845-54.
16. Huang HL, Chen YC, Huang YC, Yang KC, Pan HY, Shih SP, *et al.* Lapatinib induces autophagy, apoptosis and megakaryocytic differentiation in chronic myelogenous leukemia k562 cells. PLoS ONE 2011;6:e29014.
17. Kroemer G, Galluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, *et al.* Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. Cell Death Differ 2009;16:3-11.
18. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods 1991;139:271-9.
19. Klionsky DJ, Abdalla FC, Abeliovich H, Abraham RT, Acevedo-Arozena A, Adeli K, *et al.* Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy 2012;8:445-44.
20. Y-J Chen, S-Y Wu, C-C Chen, Y-L Tsao, N-C Hsu, Y-C Chou, *et al.* Armillaria mellea component armillarikin induces apoptosis in human leukemia cells. J Funct Foods 2014;6:196-204.
21. Huang HL, Chen CC, Yeh CY, Huang RL. Reactive oxygen species mediation of baizhu-induced apoptosis in human leukemia cells. J Ethnopharmacol 2005;97:21-9.