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Original Article

Protective Role of Comfrey Leave Extracts on UV-induced Zebrafish Fin Damage

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Abstract: In zebrafish, UV exposure leads to fin malformation phenotypes including fin reduction or absence. The present study evaluated UV-protective activities of comfrey leaves extracts in a zebrafish model by recording fin morphological changes. Chemopreventive effects of comfrey leave extracts were evaluated using Kaplan-Meier analysis and Cox proportional hazards regression. The results showed that (1) the mean times of return to normal fin in the UV+comfrey (50 and 100 ppm) groups were 3.43 and 2.86 days and were quicker compared with that in the UV only group (4.21 days); (2) zebrafish fins in the UV+comfrey (50 and 100 ppm) groups were 2.05 and 3.25 times more likely to return to normal than those in the UV only group; and (3) comfrey leave extracts had UV-absorbance abilities and significantly reduced ROS production in UV-exposed zebrafish embryos, which may attenuate UV-mediated apoptosis. In conclusion, comfrey leaves extracts may have the potential to be developed as UV-protective agents to protect zebrafish embryos from UV-induced damage. (DOI: 10.1293/tox.2013-0053; J Toxicol Pathol 2014; 27: 115–121)

Key words: comfrey, fin, UV, zebrafish

Introduction

Comfrey (*Symphytum officinale L.*) is a plant of the borage family, which is native to Europe and distributed throughout Ireland, Britain and Russia[1]. It is a fast growing plant, producing huge numbers of leaves. It is commonly used in herbal medicine and cosmetic products[2]. For example, comfrey root extract has been used for the topical treatment of painful muscle and joint complaints[3–5]. Topical comfrey creams (especially leave extracts) have been used to treat minor wounds, bruises, sprains, and varicose veins[6]. These observations suggest that comfrey has many applications, especially in terms of medical uses.

Ultraviolet (UV) radiation is a well-known environmental risk factor[7]. Inflammation, oxidative stress and DNA damage are caused by exposure to UV radiation[8, 9]. Importantly, generation of reactive oxygen species (ROS) is considered the most important adverse effect after UV exposure. In aquaculture, short-term exposure to UV radiation is used to protect juvenile fish from parasite infection[10]. However, fish exposed to excessive UV will experience some pathogenic effects, such as "solar dermatitis" and "summer lesion syndrome"[11, 12]. These observations suggest that overexposure to UV radiation is harmful to aquatic animals; in this regard, it is important to develop a low-cost and highly efficient UV-protective substance for aquaculture application.

One effective method of UV protection is enhancement of the cellular defense response by addition of ROS scavengers from natural products. Many active compounds have been proven to have UV protection activities, including (–)–epigallocatechin gallate, resveratrol, sulforaphane and flavones[13–16]. However, these active compounds are too expensive to be applied to aquaculture. Searching for a low-cost alternative is an important issue that should be addressed. Since comfrey is a fast growing plant with plenty of leaves, in this study, we used the zebrafish as a model and generated a series of time- and dose-dependent leave extracts in comfrey exposure experiments in order to evaluate their chemoprotection effects on UV-induced cytotoxicity. These results should be applicable to aquaculture.

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Materials and methods

Preparation of comfrey samples

Comfrey was kindly supplied by Yan Ten Biotech Corp, Taiwan. After eight weeks of the nutrition period, comfrey leaves were collected. The leaves were washed, air dried and ground into small particles in the presence of methanol (55 g/400 mL). The mixture was filtered to obtain a green solution. The solution was further passed through a small C-18 cartridge to remove the chlorophyll. Comfrey may contain a certain amount of pyrrolizidine alkaloids, which are capable of being removed by extraction with dichloromethane[17–18]. Finally, a powder sample was obtained by evaporation of methanol and water to dryness at room temperature in the dark. The fine particles were put into a glass bottle for further drying in the presence of phosphorus pentoxide (P_2O_5) under high vacuum for 18 h. Finally, around 1.4 g of powder were obtained and were ready for further examination.

Fish embryos culture, UV treatment and chemopreventive experiments

The procedures for zebrafish culture and embryo collection have been described previously[19]. For survival rate analysis, embryos developed at 72 hours post fertilization (hpf) were collected, randomly divided into 30 embryos per experimental group and soaked in different concentrations of comfrey leave extracts (50, 100 and 1000 ppm) without UV exposure (comfrey only) or with exposure to 302 nm UV (UVB, generated by a UV Crosslinker; Spectronics, Westbury. NY, USA) 6 times at 30-min intervals, receiving 100 mJ/cm^2 of energy each time[15]. For UV protection experiments, embryos at 72 hpf were collected, randomly divided into 3 groups (30 embryos each) and exposed to either water (UV only) or water containing comfrey leave extracts (50 and 100 ppm) in parallel to receive 100 mJ/cm^2 of UV 6 times.

Fin morphology recording and microscopy

To get a quantitative view of fin morphology, fins were compared to fins of healthy nonexperimental fish and subjectively classified as normal (at least 90% of the fin was intact), reduced (20%–90% intact) or absent (< 20% intact)[16]. All embryos were observed at specific stages under a microscope (DM 2500, Leica) equipped with Nomarski differential interference contrast optics. Photographs of embryos at specific stages were taken with a DFC490 CCD (Leica).

Detection of apoptotic cells

We performed terminal deoxynucleotidyl transferase [TdT]-mediated deoxyuridine triphosphate [dUTP] nick end labeling (TUNEL) experiments to detect apoptotic cells. By 8 dpf (5 days after exposure with UV), embryos from the mock control (fish from the same population of embryos but which were not treated with UV; no UV), UV (no comfrey; UV only), UV+50 ppm comfrey, UV+100 ppm comfrey and UV+1000 ppm comfrey groups were fixed overnight at 4 °C in 4% paraformaldehyde, and TUNEL was performed using a protocol previously reported[20–22].

Detection of ROS

To detect the accumulation of ROS in zebrafish embryos, embryos from the UV only (no comfrey) and UV + comfrey groups (50, 100 and 1000 ppm) were incubated with 500 ng/ml dihydrodichlorofluorescein diacetate (H2DCFDA, Molecular Probes). Intracellular H2DCFDA was deesterified to dichlorodihydrofluorescein, which is oxidized by ROS to produce the fluorescent compound dichlorofluorescein (DCF). After a 150-min incubation at 28 °C, the fluorescence intensity of embryos (FI) was measured with excitation/emission wavelengths of 485/530 nm. All data were presented as "ROS-scavenging rates", which were calculated with the following equation: ROS-scavenging rates (%) = $(FI_{UV+comfrey} - FI_{UV\ only}/FI_{UV\ only})*100\%$. $FI_{UV+comfrey}$ and $FI_{UV\ only}$ represent the fluorescence intensity (FI) of the UV+comfrey group and the UV only group, respectively. A positive ROS-scavenging rate indicates that treatment with the comfrey led to the generation of ROS. A negative ROS-scavenging rate indicates that the tested comfrey group has ROS-scavenging activities[20, 23].

RNA isolation and quantitative reverse transcription polymerase chain reaction (RT-PCR)

One hundred embryos derived from the UV only, UV+50 ppm comfrey and UV+100 ppm comfrey groups were collected, and their total RNAs were isolated by using the standard procedure as described previously[24–26]. Around 25 μg of total RNA from each group were used for cDNA synthesis; 1% of cDNA was used for each quantitative PCR reaction. Quantitative PCR was performed under the following conditions: 2 min at 50, 10 min at 95, and 40 cycles of 15 sec at 95 and 1 min at 60 using 2X Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and 200 nM of forward and reverse primers. Each assay was run on an Applied Biosystems 7300 Real-Time PCR System in triplicate, and fold-changes in expression were derived using the comparative C_T method (https://products.appliedbiosystems.com). An anti-apoptotic gene, *bcl2* (F, 5'-CCTTCAATAAAGCAGTGGAGGAA-3'; R, 5'-CGGGCTATCAGGCATTCAGA-3'), and several *p53*-induced apoptosis pathway-related genes, such as *p53* (F, 5'-GGCTCTTGCTGGGACATCAT-3'; R, 5'-TGGATGGCTGAGGCTGTTCT-3'), *p21* (F, 5'-CAGCTTCAGGTGTTCCTCAGC-3'; R, 5'-CGAGTGAACGTAGGATCCGC-3') and *mdm2* (F, 5'-GTGAACCAGATCGAGGACCC-3'; R, 5'-GTCAGGGAAAAGCTGTCCGA-3') were selected as targets. The β-actin (F, 5-CAGCAAGCAGGAGTACGATGAGT-3'; R, 5'-TTGAATCTCATTGCTAGGCCATT-3') was used as an endogenous control for relative quantification.

Statistical analysis

All analyses in this study were carried with the JMP statistical software (version 4.02; SAS Institute Inc., Cary, NC, USA). We treated "return to normal fin development"

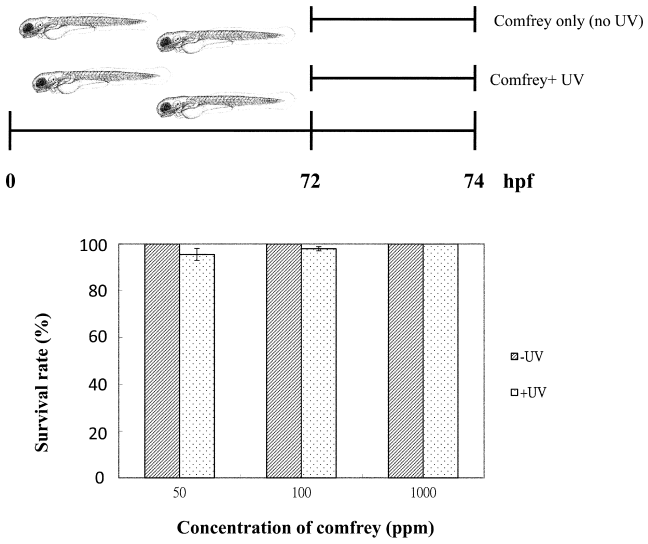

Fig. 1. Schematic representation of the experimental protocols performed in this study. Zebrafish embryos developed at 72 hours post fertilization (hpf) were collected, randomly divided into 30 embryos per experimental group and soaked in different concentrations of comfrey leave extracts (50, 100 and 1000 ppm) without UV exposure (comfrey only) or with exposure to UV (comfrey + UV).

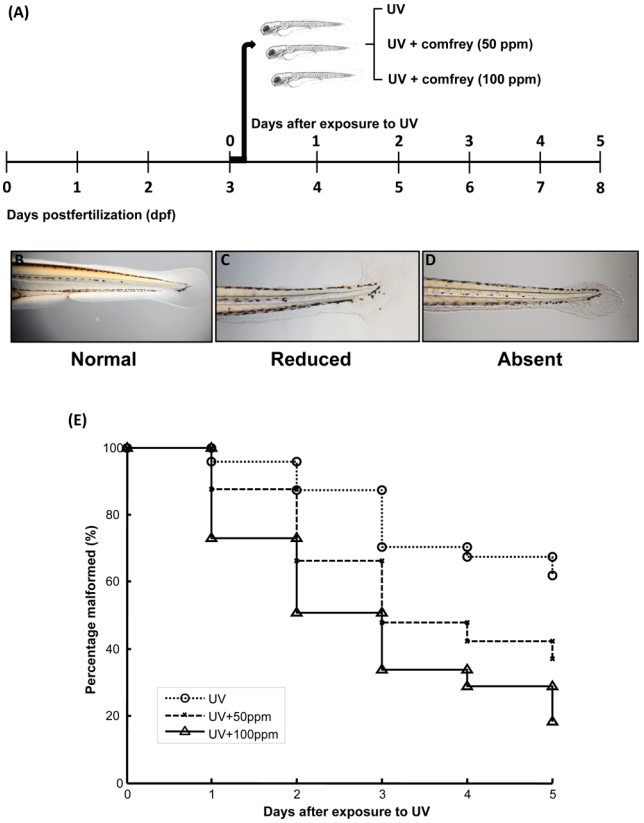

Fig. 2. UV-induced malformed fin phenotypes can be attenuated by comfrey. (A) Schematic representation of the experimental protocols performed in this study (B) Embryos display normal fins before UV exposure but exhibit reduced- (C) or absent-fin phenotypes (D) after exposure to UV. Kaplan-Meier analysis was performed to determine the number of days required for the pelvic fin to return to normal following exposure to (D) 50 and 100 ppm of comfrey leave extracts.

as the event of interest and regarded embryos that did not achieve "return" prior to death or at the end of the experiment as censored data. The Kaplan-Meier method was used to describe the malformation (non-return) rate over time and estimate the average time of return to normal for each experiment group. The log-rank test was applied to examine the difference in malformation rate between groups, and the Cox proportional hazards fit was employed to quantify the relative probability of return for each treatment group compared with the control group. The Tukey-Kramer HSD (honestly significant difference) test was used to compare the population marginal mean number of apoptotic cells for each treatment group. A significance level 0.05 was used in ANOVA analysis, and a familywise error rate of 0.05 was applied for the Tukey-Kramer HSD test.

Results

Comfrey extracts increased the rate of fin repair

Our previous studies have shown that embryonic zebrafish fins are very sensitive to UV exposure[15, 16, 21]. Thus, fin morphology has become an efficient index for evaluating UV-induced damage. In this study, we examined the preventive effect of comfrey leave extracts at different dosages on pelvic fins after UV exposure. First, we treated zebrafish embryos with different dosages of comfrey extracts (50, 100 and 1000 ppm) with or without UV exposure and calculated their survival rates. As shown in Fig. 1, there were no significant differences in survival rates between comfrey-only (100.0 ± 0%; mean ± standard error; SE) and (UV+comfrey) groups [95.7 ± 2.6% to 100.0 ± 0%; n = 30 (numbers of tested embryos in each group), N = 3 (in triplicate experiments)], suggesting that treatment with 50–1000 ppm of comfrey is not toxic to zebrafish embryos. Then, we recorded the fin morphology among all groups. As shown in Fig. 2, all of the mock control embryos (not treated with UV) displayed normal fins, but embryos exposed to UV showed a higher incidence of malformed-fin phenotypes, including fin absence or reduction. To get a better statistical point of view, we first applied the Kaplan-Meier method to describe time-to-return phenomena for each experimental group. In addition to the malformation (or non-return) rate curve (Kaplan-Meier estimate) for each group presented in Fig. 2, the mean time of return to normal and its corresponding standard error are listed in Table 1. The results revealed that UV+100 ppm comfrey experimental group had the shortest average time of return to normal (Table 1) and that the pelvic fin malformation rates, estimated 5 days after exposure to UVB, were 61.90%, 37.08% and 18.24% for the UV only, UV+50 ppm comfrey and UV+100 ppm comfrey groups, respectively (Fig. 2). We next used the log-rank test to examine the homogeneity of the malformation rate curves across the

Table 1. Summarized Results Based on the Kaplan-Meier Method for Each Experimental Group: Control (only UV), UV+50 ppm comfrey and UV+100 ppm comfrey

Experiment group	Mean time of return to normal (day)	Standard error of mean time
Only UV	4.21	0.19
UV+50 ppm comfrey	3.43	0.23
UV+100 ppm comfrey	2.86	0.24

Table 2. Cox Proportional Hazards Regression for Assessing the Effect of Comfrey Concentration on Time to Return

Experimental group	L-R chi-Square	P-value	Relative probability	Lower CL	Upper CL
UV+50 ppm comfrey	5.28	0.022	2.05	1.11	3.90
UV+100 ppm comfrey	16.84	0.000	3.25	1.83	6.04

CL: confidence limit.

groups. The result showed a significant difference in time-to-return among these groups (p-value < 0.0001), confirming that UV+100 ppm comfrey experimental group had a significantly optimal repair effect.

The Cox proportional hazards regression analysis (Table 2) demonstrates that the relative probabilities of return to normal fin (with corresponding confidence limits) for the UV+50 ppm comfrey and UV+100 ppm comfrey groups compared with control (UV only) group were 2.05 (1.11–3.90) and 3.25 (1.83–6.04). The former indicates with statistical significance (p-value=0.022) that a zebrafish in the UV+50 ppm comfrey group was 2.05 times more likely to achieve return than one in the UV only group. The latter significantly suggests that a zebrafish in the UV+100 ppm comfrey group was 3.25 times more likely to achieve return than one in the control group (p-value=0.000). This indicates that the comfrey extracts increased the rate of fin repair in a dose-dependent manner.

Comfrey protects zebrafish larvae from UV-mediated fin damage by preventing apoptosis of cells

It has been demonstrated that UV-induced zebrafish fin damage is due to apoptosis[23]. Our data demonstrated that the UV-induced malformed fin phenotypes can be attenuated by co-exposure to comfrey leave extracts (Fig. 2). Here, we carried out a TUNEL assay to further confirm whether comfrey leave extracts can protect cells from UV-induced apoptosis. The results showed that no apoptotic signals were observed in the embryos derived from the no UV group (Fig. 3A), but many apoptotic signals accompanying malformed fin phenotypes were found in the embryos after exposure to UV (UV only group; indicated by an arrow in Fig. 3B). However, few or no signals were found when these embryos were co-exposed to UV with 50–1000 ppm of comfrey extracts (Figs. 3C–3E). To pinpoint which treatment means were significantly different from each other, the Tukey-Kramer HSD test was further used for pairwise comparisons. Figure 3F presents the mean numbers and their 95% confidence intervals for the five treatment groups. The test revealed that the mean numbers for the no UV, UV only, UV+50 ppm comfrey, UV+100 ppm comfrey and UV+1000 ppm comfrey groups were 13.67, 162.50, 93.33, 56.03 and 7.57, with the common standard error being 2.82, and also identified that the mean numbers for the five treatment groups were significantly different from each other, except those for the no UV and UV+1000 ppm comfrey groups (Fig. 3F). This indicates that the UV+1000 ppm comfrey group had the potential to let the UV-treated zebrafish fins return to normal. Thus, we propose that comfrey extract has a chemoprevention ability that protects UV-damaged fin cells from apoptosis.

The ROS-scavenging and UV-absorbance abilities of comfrey leave extract may contribute to its UV-protection efficiency

Previous studies have shown that UV exposure is associated with the generation of ROS[20, 27]. In this study, we detected the level of ROS in zebrafish embryos treated with UV and 50–100 ppm of comfrey leave extracts. As shown in Fig. 4, the level of ROS in zebrafish embryos treated with comfrey extract was decreased in a concentration-dependent manner, with 51.7%, 82.8% and 93.1% decreases for the 50, 100 and 1000 ppm comfrey extract treatments compared with the UV only group (no comfrey). For the UV absorbance experiment, 0, 50 and 100 ppm of comfrey leave extracts were used to measure the absorbance between 280–410 nm. As shown in Fig. 5, comfrey extracts indeed had photochemical properties, especially in the wavelength range of 290–340 nm. These data demonstrated that the ROS-scavenging and UV-absorbance abilities of comfrey leave extract may contribute to its UV-protection efficiency.

Possible mechanisms of chemoprevention of UV-induced fin damage by comfrey

From the molecular point of view, UV-induced cell apoptosis has been shown to accumulate the expression of *p53* and its downstream target, *p21*. *mdm2* is a negative regulator of *p53*, whereas *bcl2* is a cell cycle regulator proteins that is thought to have anti-apoptotic activity[28]. In this study, we carried out quantitative RT-PCR experiments to further investigate the molecular mechanisms for chemoprevention of UV-induced fin damage by comfrey extract. As shown in Table 3, the expression levels of *p53* and *p21* in the embryos derived from UV+comfrey (50 and 100 ppm) groups increased by 1.4- to 2.7-fold, in comparison with those of embryos derived from the UV only group; the expression levels of *mdm2* were downregulated by 0.6-fold. This suggests that comfrey treatment might induce the p53-related pathway. However, the expression levels of *bcl2* were increased by 1.2- to 1.5-fold. Taken together, we propose that comfrey may increase the expressions of *bcl2* to protect fin cell UV-induced apoptosis.

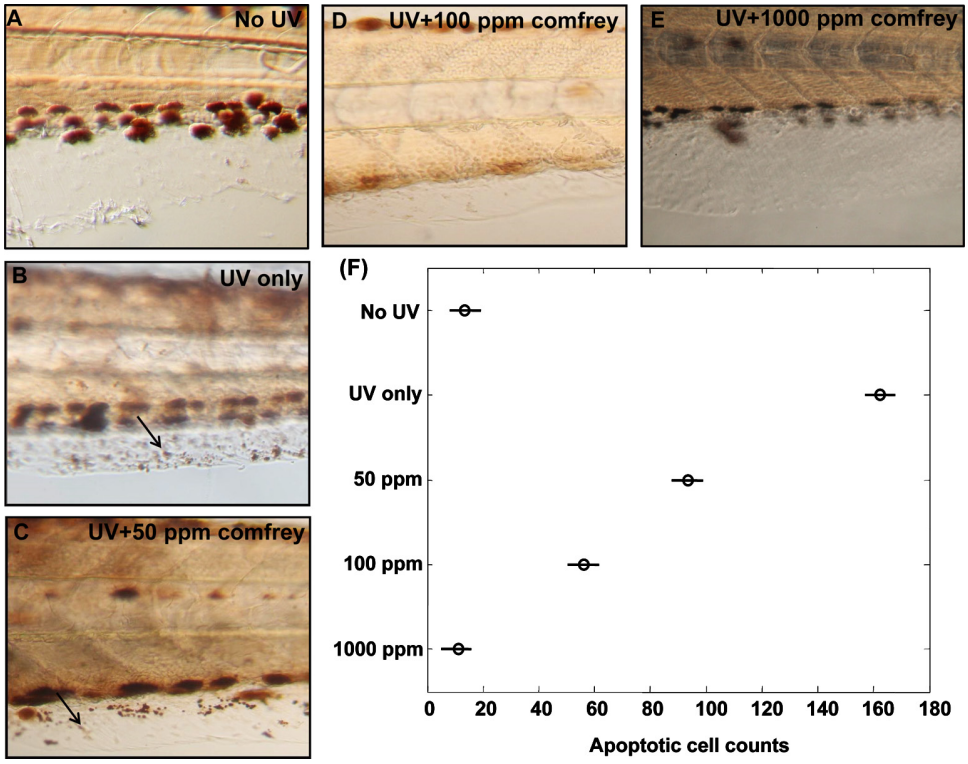

Fig. 3. UV exposure results for cell apoptosis in the fin region. Lateral views of mock control embryos without (A) and with UV exposure (B) after TUNEL assay staining. (C) Lateral views of embryos derived from the UV+50 ppm comfrey group, (D) UV+100 ppm comfrey group or UV+1000 ppm comfrey group (E) after TUNEL assay staining. Arrows indicate the apoptotic cells. (F) The Tukey-Kramer HSD (honestly significant difference) test reported the marginal mean cell counts and corresponding 95% confidence intervals for all groups. The means of two groups are significantly different if their intervals are disjoint and are not significantly different if their intervals overlap.

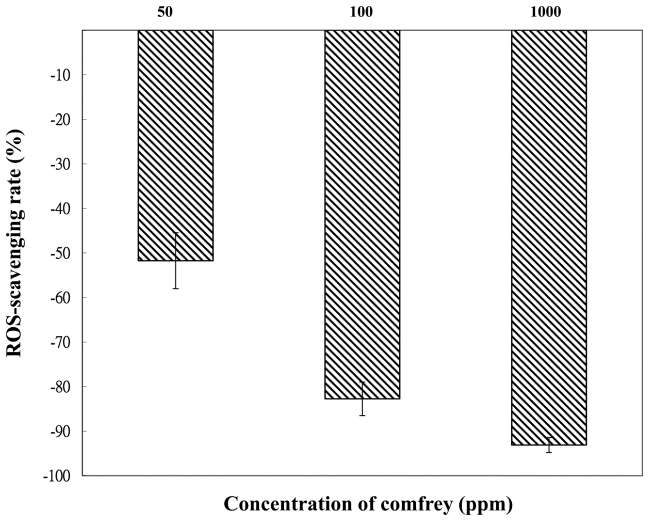

Fig. 4. Repression of UV-induced ROS production by comfrey. UV-induced ROS levels are regulated by comfrey. The ROS levels were measured using the oxidant-sensitive probe H2DCFDA. The X- and Y-axes represent the different concentrations of comfrey and ROS-scavenging rates, respectively. ROS-scavenging rates were calculated using the following equation: ROS-scavenging rates (%) = $(FI_{UV+comfrey} - FI_{UV\ only} / FI_{UV\ only}) * 100\%$.

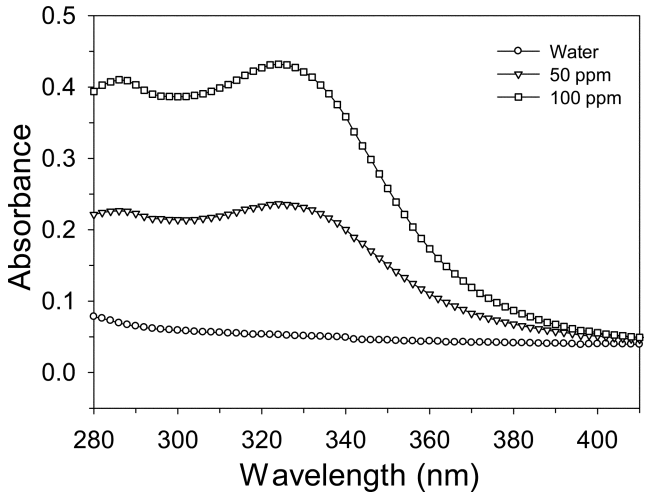

Fig. 5. Absorbance spectrum of comfrey leave extract between 280-410 nm. Comfrey leave extracts of 0 (circle), 50 (triangle) and 100 (square) ppm were used to measure the absorbance between 280–410 nm, respectively. The instrument used was a JASCO V-550 UV/VIS spectrophotometer, and a quartz cuvette was used. The path length was 1 cm.

Table 3. Relative Quantification of *p53, p21, bcl2* and *mdm2* Expression Levels Using the Comparative C_T Method

Group	Target Average C_T	β-actin Average C_T	ΔC_T Target-(β-actin)	$\Delta\Delta C_T$ $\Delta C_{T(experimental)}$-$\Delta C_{T(control)}$	Fold changes relative to control group[#]
p53					
UV	30.28 ± 0.51	25.10 ± 0.31	5.186	0	1
UV + 50 ppm comfrey	29.65 ± 0.24	25.14 ± 0.28	4.509	–0.678	1.599
UV + 100 ppm comfrey	30.17 ± 0.47	25.79 ± 0.14	4.376	–0.810	1.753
p21					
UV	31.39 ± 0.60	25.10 ± 0.31	6.289	0	1
UV + 50 ppm comfrey	30.91 ± 0.01	25.14 ± 0.28	5.767	–0.522	1.436
UV + 100 ppm comfrey	30.64 ± 0.27	25.79 ± 0.14	4.846	–1.443	2.720
bcl2					
UV	31.29 ± 0.42	25.10 ± 0.31	6.197	0	1
UV + 50 ppm comfrey	31.03 ± 0.58	25.14 ± 0.28	5.888	–0.309	1.239
UV + 100 ppm comfrey	31.34 ± 0.18	25.79 ± 0.14	5.547	–0.650	1.569
mdm2					
UV	29.40 ± 0.50	25.10 ± 0.31	4.303	0	1
UV + 50 ppm comfrey	30.12 ± 0.04	25.14 ± 0.28	4.974	0.671	0.628
UV + 100 ppm comfrey	30.76 ± 0.19	25.79 ± 0.14	4.964	0.661	0.632

C_T: cycles of qPCR. [#]Relative folds to control group=$2^{-\Delta\Delta CT}$.

Discussion

In this study, we demonstrated that fin damage in zebrafish embryos caused by UV can be attenuated by treatment with comfrey leave extracts. In order to apply comfrey extracts to aquaculture and fish physiology, the toxicants of the comfrey extracts should be removed. It was reported that comfrey contains dangerous levels of toxic pyrrolizidine alkaloids and that its use led to severe liver injury and death[29–32]. Because of its toxicity, comfrey (leaves and roots) crude extracts have often been processed as topical cream, and it has been recommended that they never be taken by mouth or even applied comfrey to broken skin[1, 33]. In general, the root of the plant contains more pyrrolizidine alkaloids than the leaves. To avoid the poison effect of pyrrolizidine alkaloids, we selected comfrey leave extracts as materials and used a pyrrolizidine alkaloid-free purification protocol. Our study indicated that treatments with 50–1000 ppm of purified comfrey leave extract are not toxic to zebrafish embryos (Fig. 1). Thus, comfrey leave extracts might have the potential to be applied to aquaculture research.

From the molecular points of view, P53 and Bcl2 are important regulators of the cell cycle and cell apoptosis. It has been reported that Bcl2 can constitutively suppress p53-dependent apoptosis[28]. Thus, Bcl2- and P53-related pathways might be close to each other but function independently. Our data showed that comfrey treatment enhances the expressions of *p53* and *bcl2* and consequently protects zebrafish fins from UV-induced damage. However, in some cases, addition of an oxidant (e.g., resveratrol) can increase the expression of P53[34]. In this regard, the increased expressions of *p53* and *bcl2* might be from the addition of comfrey extracts. In addition, our study clearly showed that comfrey leave extracts have dose-dependent ROS-scavenging and UV-absorbance activities (Figs. 4, 5). Taken together, we propose that the UV-protective ability of comfrey extract may mostly come from its photochemical properties, which can isolate UV. That is, comfrey leave extracts may act just like a sunscreen, providing protection against UV-induced fin damage from the extracellular level. In summary, this study suggests that comfrey can be used to protect zebrafish fins from UV-induced damage, implying that it may be applied to aquaculture to enhance the survival of juvenile fish.

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