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Original Article

In vivo pro-angiogenic effects of dracorhodin perchlorate in zebrafish embryos: A novel bioactivity evaluation platform for commercial dragon blood samples

Preethi Krishnaraj [a], *Yu Chang* [b], *Tsung-Jung Ho* [c,d,e], *Nai-Chen Lu* [e,f], *Ming-Der Lin* [a,g,h,**], *Hao-Ping Chen* [b,*]

[a] *Department of Molecular Biology and Human Genetics, Tzu Chi University, Hualien, 97004, Taiwan*
[b] *Department of Biochemistry, Tzu Chi University, Hualien, 97004, Taiwan*
[c] *College of Chinese Medicine, China Medical University, Taichung, 40421, Taiwan*
[d] *Division of Chinese Medicine, China Medical University Beigang Hospital, Yulin, 65152, Taiwan*
[e] *Division of Chinese Medicine, An Nan Hospital, China Medical University, Tainan, 70965, Taiwan*
[f] *Department of Nursing, An Nan Hospital, China Medical University, Tainan, 70965, Taiwan*
[g] *Department of Life Science, Tzu Chi University, Hualien, 97004, Taiwan*
[h] *Department of Medical Research, Hualien Tzu Chi Hospital, Hualien, 97004, Taiwan*

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ABSTRACT

Dragon blood has been used in wound treatment for many years and can be obtained from several distinct plant species. Dracorhodin, the active substituent of dragon blood, is a characteristic compound of the palm tree, *Daemonorops draco*. At present, the only method to evaluate the quality of commercial dragon blood samples is a HPLC method which determines the amount of dracorhodin in a dragon blood sample. In this study, we used zebrafish embryos as a platform to demonstrate the *in vivo* pro-angiogenic activity of dracorhodin perchlorate, the chemically synthesized analog of dracorhodin. By using this platform, three different commercial dragon blood samples were also examined. Our results clearly show that even though the commercial dragon blood samples had similar amounts of dracorhodin, they showed highly variable biological activity, such as pro-angiogenic effects and toxicity. In short, an *in vivo* activity assay platform for rapidly examining the biological activity of commercial dragon blood samples was successfully established here, which complements the current HPLC-based assay method.

* *Corresponding author*. Department of Biochemistry, Tzu Chi University, 701, Sec 3, Zhongyang Road, Hualien, 970, Taiwan. Fax: +886 3 8580641.
** *Corresponding author*. Department of Molecular Biology and Human Genetics, Tzu Chi University, Hualien, Taiwan. Fax: +886 3 8578386.
E-mail addresses: mingder@mail.tcu.edu.tw (M.-D. Lin), hpchen@mail.tcu.edu.tw (H.-P. Chen).
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1. Introduction

Dragon blood has been used in traditional medicine worldwide for many years. However, "dragon blood" can be obtained from several distinct plant species. Dragon blood from Socotra Island in the Arabian Sea, Yemen, is produced from the red resin of *Dracaena cinnabari*. It is generally believed that Socotra dragon blood was imported to China through the Silk Road during the Tang and Song Dynasties (between 618 AD and 1279 AD). Another type of dragon blood is produced from fruit sap of the palm tree, *Daemonorops draco*. This plant is mainly distributed in Malaysia and Sumatera Island, Indonesia. Due to the proximity of South-East Asia to China and advances in navigation technology, palm tree dragon blood became the major product in the traditional Chinese medicine market after the Ming Dynasty (between 1368 AD and 1644 AD). Europeans also found dragon blood alternatives in the Amazon basin after Columbus reached the New World. The red resin can be collected from the trunk of some plant species such as *Croton salutaris*, *C. lechleri*, and *C. draconoides*. More recently, a new indigenous species of dragon blood tree, *Dracaena cochinchinensis*, was discovered by the Chinese botanist, Xitao Cai, in Yunnan Province, China in 1972. Since then, domestic dragon blood products have appeared in China's traditional medicine market [1,2].

In current traditional Chinese markets in Taiwan, palm tree "dragon blood" is the mainstream product. It can be classified into two different categories by its appearance, powder form and block form. The powder form is a dried powder obtained from the red fruit resin of the palm tree, *D. draco*, without other artificial additives (Fig. 1A and B). On the other hand, the block form dragon blood is a dark brown subglobular block with a diameter of 6–8 cm (Fig. 1C–F). For the purpose of transport and storage, the powder from fruit resin is mixed with an excipient, dammar gum, to obtain the solid. It is generally believed that dammar gum protects the active compounds inside the block from moisture and oxygen, extending the shelf life of the product. The extended shelf life of the block form may be why it is the main retail product sold in the traditional Chinese medicine market.

The biological activities of dragon blood have been evaluated in cell-based culture systems. The crude dragon blood extract from *D. draco* can decrease vascular inflammation and promotes osteoblast differentiation, mineralization, antiplatelet aggregation, and bone formation [3–5]. Similarly, pressure ulcers patients can be successfully treated by crude extracts [6]. These results are consistent with the description of dragon blood in ancient traditional Chinese medical literature. While dracorhodin is the characteristic compound of palm tree dragon blood, its chemically synthetic analog, dracorhodin perchlorate (DP) showed both angiogenic activity on HUVEC cells in a cell-based two dimensional tube formation assay and wound healing activity in animal studies [7,8].

While the angiogenic and wound healing effects of dragon blood have been demonstrated, an efficient *in vivo* platform for evaluating the bioactivity of dragon blood samples has yet to be established. Since genetic and pharmacological evidence shows that the vascular biology of zebrafish is comparable to that of humans, zebrafish has recently become a promising animal model to study angiogenesis [9,10]. Furthermore, the rapid embryonic development and transparency of fish embryos allows the angiogenic dynamics and the entire process of *de novo* or re-vascularization to be visualized and quantified rapidly without disrupting or sacrificing animals. In contrast, the *in vitro* tube formation assay, a cell-based method used for screening compounds that possess angiogenic activity, has several disadvantages. For example, it is not suitable for evaluating sprouting angiogenesis, new blood vessel formation from pre-existing major donor vessels [11]. Moreover, the tubes formed in the cell based assay may not reflect real capillaries as they lack lumen in early stages [12]. Here, we report and compare the *in vivo* angiogenic activity of DP and commercially available dragon blood products by using a newly developed zebrafish embryo assay platform.

2. Materials and methods

2.1. Materials

Drachorodin perchlorate (DP) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). HPLC grade acetonitrile was obtained from ECHO Chemical Co., LTD (Miaoli, Taiwan). Molecular biology grade DMSO, Pronase (catalog number: PRON-RO), and 1-phenyl-2-thiourea (PTU) were from Sigma–Aldrich (St. Louis, MO, USA). Vivaspin 500 Protein Concentrators (MWCO 3000 and 30,000) were from GE Healthcare Life Science (Taipei, Taiwan) Both block forms of dragon blood, Baochu brand and Golden Chicken brand, were purchased from a traditional Chinese medicine store in Taipei. Powder form dragon blood was imported from Indonesia (Fig. 2 and Table 1).

2.2. Zebrafish maintenance and chemical treatment

The zebrafish *Danio rerio* AB strains were maintained at 28 °C with a 14 h dark and 10 h light cycle. Two males and four females were set-up for breeding. The embryos obtained were maintained and raised in E3 buffer (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$, pH 7.2) throughout the experimental procedure at 28 °C with the same photoperiod as that of the adults. All the embryos used for experiments were *Tg(fli1-EGFP)* [13] unless otherwise specified. Dechorionation was performed by treating with pronase (1 mg/ml) at 12 h post-fertilization (hpf). The dechorionated embryos was then treated with PTU (1.5 mg/ml) at 24 hpf to inhibit pigmentation. The embryos were transferred to a 12-well plate at 55 hpf, with each well containing five embryos. DP or crude dragon blood extracts in DMSO were diluted in E3 buffer at the specified concentration indicated and the final working solution contained 0.1% DMSO. Observations for mortality and angiogenic phenotypes were made at 72 hpf and images were captured using a fluorescent stereomicroscope (Olympus SZX7) equipped with a CCD camera. Phenotypic quantifications for the sprout length were performed using Image J (http://imagej.nih.gov/ij/). Data was analyzed by one-way ANOVA followed by Dunnet's multiple comparison, using GraphPad Prism (San Diego, CA). Institutional Animal Care and Use Committee of Tzu Chi University approved all animal experiments in this study (Case number: 106,064).

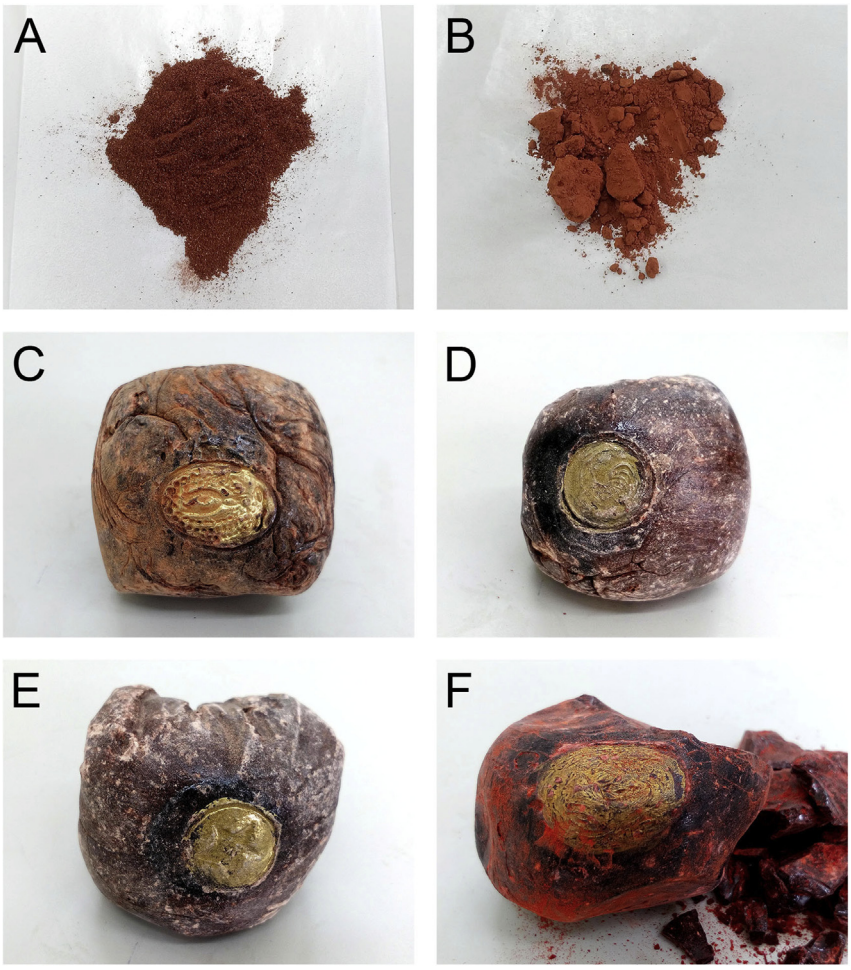

Fig. 1 – Appearance of powder form and block form dragon blood. Block form dragon blood is made by mixing red tree resin with dammar gum. Powder form dragon blood (Sample A and B). Block form dragon blood (Sample C, D, E, and F).

2.3. *HPLC analysis for dragon blood*

HPLC experiments were carried out on a Hitachi L-7000 HPLC system, equipped with a L-7100 quaternary gradient pump and a L-7450 photo diode array detector. Hitachi HSM software was used for machine controlling, data collecting, and processing. A Mightysil, RP-18, 5 μm, 250 × 4.6 mm, analytic column (Kanto Chemical Co., Inc., Tokyo, Japan) was used for analysis. The HPLC separation conditions (in supporting information, Figure S1) were reported previously [14].

2.4. *Preparation of dragon blood DMSO crude extracts for determination of angiogenic active compound molecular weight ranges in dragon blood*

1 g powder form dragon blood, sample A, (dracorhodin content: 2.4%) was weighed and dissolved in 10 ml of 95% ethanol. After soaking at room temperature for two days, 1 ml extracts were dried under vacuum and re-suspended in 1 ml 50% ethanol. After cleared by centrifugation, the white insoluble pellet was collected and 0.5 ml supernatant was loaded onto Vivaspin 500 Protein Concentrators (MWCO 3000 or 30,000), and centrifuged at 14,000× g for 10 min. Collected filtrates were washed in the concentrators with another 0.5 ml fresh 50% ethanol twice, and centrifuged again. Pooled filtrates were dried by a SpeedVac concentrator (Thermo Fisher Scientific, Waltham, MA, USA), and re-dissolved in 250 μl DMSO for angiogenesis activity assays. The dracorhodin concentration in this DMSO stock should be 12 mg/ml.

2.5. *Preparation of dragon blood DMSO crude extracts for angiogenesis activity assays*

Ground solids (1.0 g) were weighed and dissolved in 100 ml of methanol. After soaking at room temperature overnight, methanol extracts were transferred to a new glass vial by using disposable glass Pasteur pipettes. Another 40 mL of methanol was added and extracted at room temperature overnight. The final volume of the extract was adjusted to 250 mL by adding methanol. Undissolved particles were removed by centrifugation at 2500× g for 10 min at room temperature and filtrated through a 0.22 μm syringe filter. The dracorhodin concentration in the methanol extract was then determined by HPLC. Methanol extracts containing 2.5 mg dracohordin were evaporated to dryness under vacuum. To remove minor residual water and methanol, the extract was further dried by freeze-drying. The dried powder was dissolved in DMSO to a final volume to 1.0 ml. The un-dissolved white gum dammar was discarded.

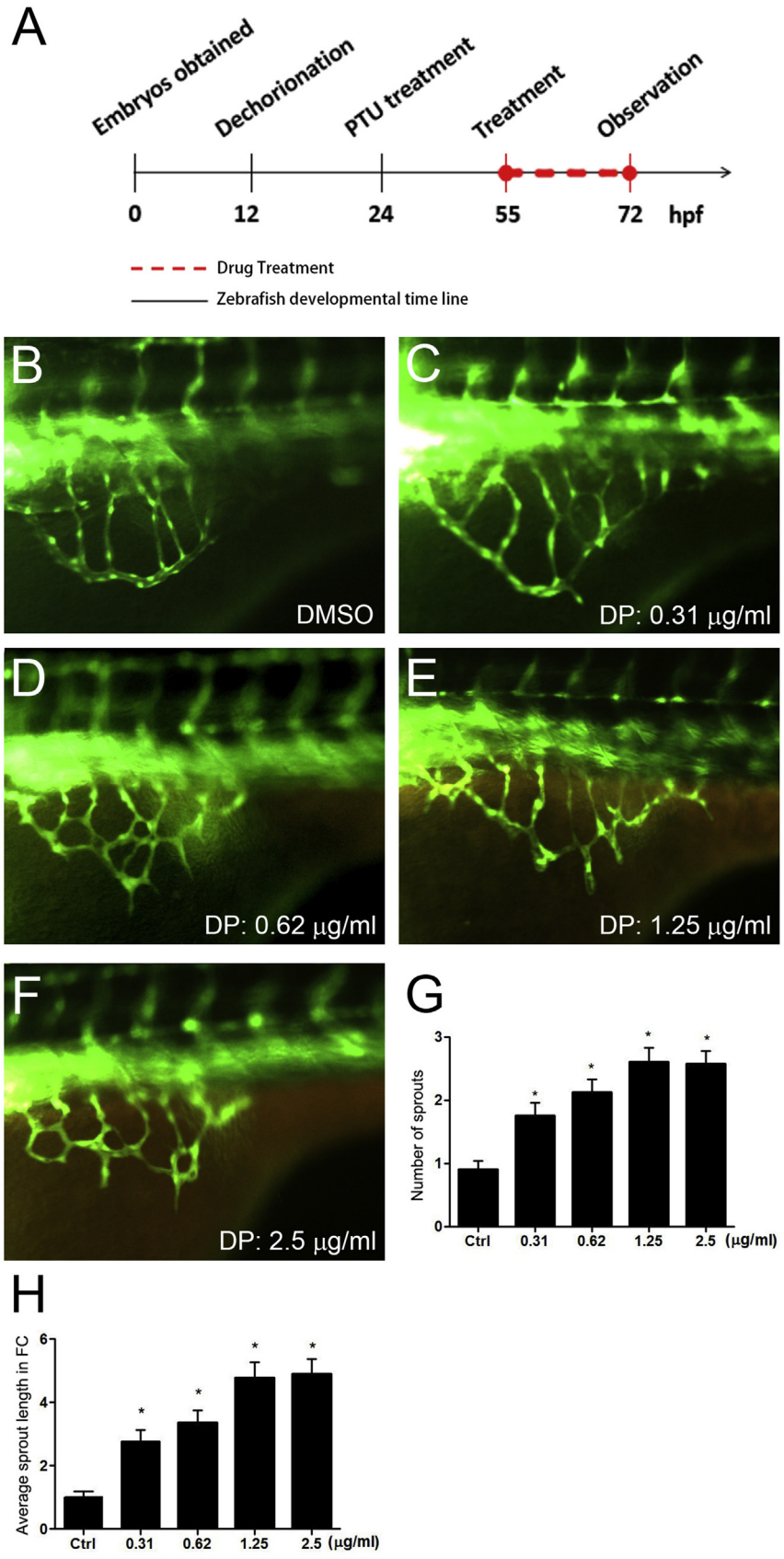

Fig. 2 – Effect of Dracorhodin perchlorate on sub-intestinal veins of zebrafish embryos. (A) Time line of zebrafish embryonic development showing the experimental procedures at different time points. (B) Sub-intestinal veins (SIV) of control embryo treated with 0.1% DMSO. (C–F) SIV of 72 hpf zebrafish embryos treated with (C) 0.31, (D) 0.62, (E) 1.25, and (F) 2.5 μg/ml of

Table 1 – Commercial dragon blood samples used in this study.

Dragon blood samples	Commercial name	Place of origin	Dracorhodin content
Sample A	N/A	unknown	2.4%
Sample B	N/A	Medan, Indonesia	1.2%
Sample C	Baochu	Singapore	0.96%
Sample D	Golden Chicken	Singapore	0.02%
Sample E	Golden Star	Singapore	0.02%
Sample F	B.B. Crown	unknown	Not detected

3. Results and discussions

3.1. *DP-induced pro-angiogenic effects on zebrafish embryos*

To determine the angiogenic properties of drachorodin perchlorate, zebrafish embryos were treated with drachorodin perchlorate (DP) from 55 to 72 hpf and sub-intestinal vein (SIV) development was examined at 72 hpf (Fig. 2A). This time frame marks the onset of the second phase of SIV development to the completion of vascularization in SIVs [15]. Compared to the vehicle control with 0.1% DMSO (Fig. 2B), DP treated embryos exhibited a pro-angiogenic phenotype, showing the formation of extra sprouts (Fig. 2C–E, arrowheads). To further quantify the newly formed sprouts, the average number of sprouts and the corresponding length was measured (Fig. 2F and G). The average number of sprouts in the DP treated embryos increased in a dose-dependent manner and was statistically significant when compared to the control embryos (Fig. 2F). Additionally, the relative fold changes in the sprout length with respect to controls in the DP treated groups also increased significantly (Fig. 2G). We also noticed that the effect of DP treatment plateaued at 1.25 and 2.5 μg/ml as shown by average sprout numbers and length. Although the survival rate of DP treated embryos at 72 hpf were 100%, about 5% of the embryos treated with 2.5 μg/ml of DP presented mild cardiac edema. In contrast, the embryos treated with 1.25 μg/ml of DP did not present gross morphological defects including cardiac edema, local motion, and other morphological deformities. We, therefore, treated the embryos with 1.25 μg/ml of DP in the following experiments.

3.2. *Determination of dracorhodin content in commercially available samples and molecular weight ranges of angiogenic active compounds*

HPLC analysis was carried out to determine dracorhodin content in dragon blood samples purchased from a traditional Chinese medicine store. Perhaps not surprisingly, the dracorhodin concentration in the powder form of dragon blood (Sample A and B) is significantly higher than that in the block form of dragon blood (Sample C, D, E, and F). However, the dracorhodin concentration of both Samples D and E was only 0.02%, and could be barely detected in Sample F (Table 1), far below the standard (1.0%) set by official documents in China, Hong Kong, and Taiwan [16–18]. Therefore, these three brands (Sample D-F) did not qualify and were excluded from further studies. Since, it is still not clear whether dracorhodin is the only compound that possesses angiogenic activity in crude dragon blood, the molecular weight range of potential angiogenic active compounds was investigated. Because of the solvent restriction of membrane for molecular weight fractionation, 50% ethanol is used as the solvent for fractionation. Ethanol extracts of crude dragon blood were fractionated by a spin column fitted with a membrane of molecular weight cut off of 3000 or 30,000 Da. The angiogenic activity of the two different filtrates was examined. No significant difference can be found among these three groups when comparing sprout number to sprout length in SIV (results in supporting information, Figure S2). These results suggest that the molecular weight of angiogenic active compounds is less than 3000 Da, despite the presence of many high molecular weight bio-polymers in plant resin.

3.3. *Toxicity of crude dragon blood extract on zebrafish embryos*

To systematically investigate the possible toxic effects of crude dragon blood extracts, embryos were exposed to dragon blood extracts containing 0.31 μg/ml, 0.63 μg/ml, 1.25 μg/ml, and 2.5 μg/ml of dracorhodin (Fig. 3). The procedure of embryos treatment was the same as the aforementioned experiments. The high lethality of the embryos from Sample B particularly caught our attention as this indicates the presence of unidentified substances that are toxic to the zebrafish embryos. Since we have equalized the dracorhodin content in these samples, the unknown toxic compound could not be dracorhoin. Also, it is not clear whether other commercial samples contain this toxic molecule or not.

3.4. *Comparison of angiogenic sprouting on the treatment of DP and crude dragon blood extracts*

To further investigate whether dracorhodin is the only compound responsible for the pro-angiogenic effects on

dracorhodin perchlorate (DP). White arrows indicate extra sprouts. (G) Quantification of average sprout numbers. DP increased the average sprout number in a dose-dependent manner between 0.31 and 1.25 and plateaued at 1.25–2.5 μg/ml. (H) Quantification of relative fold changes in sprout length with respect to controls. DP increases the sprout length in a dose-dependent manner. Data is expressed as a mean ± SEM from three independent experiments. Asterisks indicates $P < 0.05$ compared with the control group.

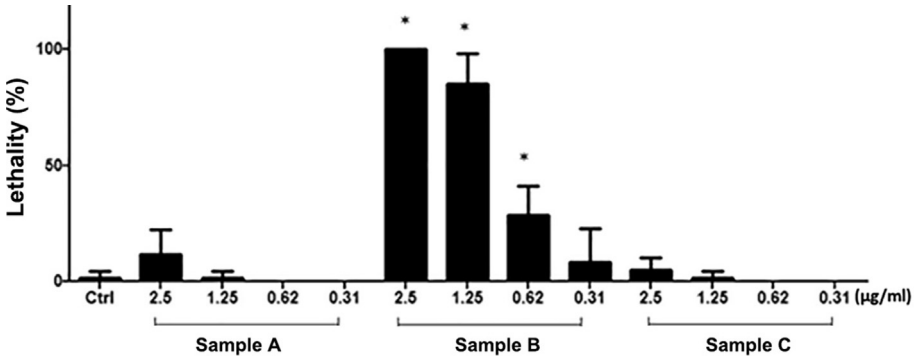

Fig. 3 – Toxicity of crude dragon blood extracts on zebrafish embryos. Lethality of embryos at 72 hpf treated by DP or crude extracts of sample A, B and C. Data is expressed as a mean ± SEM from three independent experiments. *P < 0.05 compared with 0.1% DMSO treated controls.

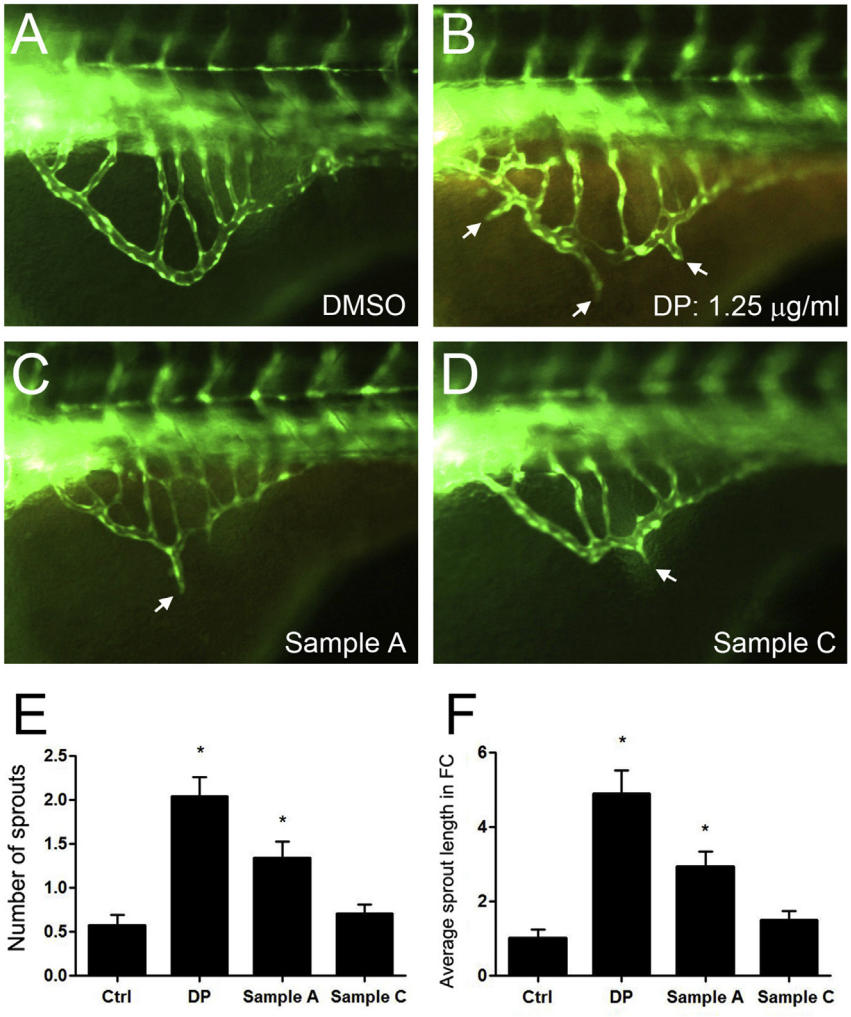

Fig. 4 – Comparisons of vascular phenotypes between commercially available dragon blood and DP. (A) SIV of an embryo treated with 0.1% DMSO. (B) SIV of an embryo treated with 1.25 μg/ml DP. (C) SIV of an embryo treated with a crude extract of Sample A containing 1.25 μg/ml dracorhodin. (D) SIV of an embryo treated with a crude extract of Sample C containing 1.25 μg/ml dracorhodin. (E) Quantification of average sprout number. (F) Relative fold changes in the sprout length with respect to controls. White arrows indicate extra sprouts. Quantification of vascular phenotypes expressed as a mean ± SEM. Asterisk indicates P < 0.05 compared with the control group.

zebrafish embryos, we examined and compared the SIV phenotypes of crude methanol extracts from Sample A and Sample C containing 1.25 μg/ml dracorhodin with that of 1.25 μg/ml DP. Our results showed that both DP (Fig. 4B) and crude extract from Sample A (Fig. 4C) produced extra sprouts with longer lengths compared to vehicle controls (Fig. 4A,E,F). However, there was no significant change in either the sprout number or the sprout length from the crude extract of Sample C (Fig. 4D) compared with the vehicle controls (Fig. 4A,E,F). As the treated crude extracts had been adjusted to possess the same amount of dracorhodin, our result suggests that dracorhodin might be not the only compound in crude dragon blood that can modulate the pro-angiogenic effects. It is also noteworthy that the sprout number in the DP-treated group is significantly higher than the crude dragon blood extract-treated group (Fig. 4E and F). DP is a synthesized chemical analog of dracorhodin. Because dracorhodin is not commercially available at this moment, it is unclear whether DP has more potent angiogenic activity than dracorhodin. Moreover, DP carries one more positive charge than dracorhodin, which might lead to greater solubility. The solubility of dracorhodin might be therefore significantly altered by the presence of other components in crude dragon blood extracts.

4. Conclusion

The use of dragon blood in wound treatment has been recorded in many ancient traditional Chinese medical books such as Preions for Universal Relief (published in 1390 AD), Compendium of Materia Medica (published in 1596 AD), Golden Mirror of Medicine (published in 1742 AD), and Compendium of Wound Specialist (published in 1760 AD). Although dragon blood has been used for wound treatment worldwide for many years, quality control and evaluation has always been a major concern for this natural medical product. Routine examination of dragon blood depends on the HPLC analysis of the dracorhodin content, as described in pharmacopeias [16–18]. As shown in our results, only three out of six commercial dragon blood samples met the standard of pharmacopeias. Furthermore, our results clearly show that the biological activity (pro-angiogenic effects and toxicity) could vary quite a lot in different dragon blood samples, even though their dracorhodin content was the same. Accordingly, the content of dracorhodin in commercial dragon blood samples is not accurate enough to be used as the only quality evaluation standard. An *in vivo* activity assay platform for rapid examination of the biological activity of commercial dragon blood samples was successfully established in this study.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jfda.2018.08.005.

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