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Anti-angiogenic effects of rhubarb and its anthraquinone derivatives

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ABSTRACT

Ethnopharmacological relevance: Rhubarb root (Dahuang) is often included as an ingredient in traditional Chinese compound prescriptions for the treatment of inflammatory diseases. This application may possibly be mediated through anti-angiogensis and thus would shed light on its potential value in cancer therapy.
Aim of the study: To elucidate the anti-angiogenic properties of rhubarb root, we tested the inhibitory effects of different fractions and a series of anthraquinone derivatives against vessel formation in zebrafish embryos.
Materials and methods: The 95% ethanol extract and four subsequent fractions (*n*-hexane, ethyl acetate, *n*-butanol and aqueous fractions) of rhubarb root and five anthraquinone derivatives were investigated on zebrafish model by quantitative endogenous alkaline phosphatase assay and staining assay.
Results: Ethyl acetate fraction showed the strongest inhibition of vessel formation by 52%. Three anthraquinones (aloe-emodin, emodin and rhein) displayed potent anti-angiogenic activities.
Conclusions: The angiogenic properties of rhubarb root may partly account for its use in inflammatory diseases. The anthraquinones with acidic or polar, hydrophilic substitution at C-6 or C-3 positions played a substantial role in inhibiting angiogenesis. The value of the zebrafish angiogenic model is further supported.

1. Introduction

Rhubarb root (Dahuang), one of best-known traditional Chinese medicine, has been widely used for thousands of years in China for the treatment of many diseases, including constipation, jaundice, gastrointestinal hemorrhage, and ulcers (Chinese Pharmacopoeia Commission, 2005). On the other hand, it is often included as an ingredient in traditional Chinese compound prescriptions for the treatment of inflammation, such as acute appendicitis, acute cholecystitis and rheumatoid arthritis (Qiu, 1994; Anon., 1976). In recent years, rhubarb has been shown to have good antitumor (Dorsey and Kao, 2007; Huang et al., 2007; Lee et al., 2001; Shi et al., 2001) and anti-inflammation effects (Cuellar et al., 2001).

The role of inflammation in the promotion of carcinogenesis was originally proposed by Virchow in 1863. Chronic and persistent inflammation contributes to cancer development and may be responsible for a substantial portion of tumor vascularization in "inflammatory angiogenesis", indicating that combating inflammation with appropriate drugs or substances could prevent inflammatory angiogenesis in carcinogenesis (Albini et al., 2005; Allavena et al., 2008; Bisacchi et al., 2003). Angiogenesis refers to the formation of new blood capillaries from pre-existing ones, and is essential in a series of normal physiological processes such as embryonic development and pathological responses. However, persistent unregulated angiogenesis would cause 'angiogenic diseases' such as diabetic retinopathy, tumor growth and metastasis, rheumatoid arthritis, and inflammatory diseases (Folkman, 1995). Folkman (1971) was first to hypothesize a linkage between angiogenesis, tumor growth and metastasis, and the inhibition of angiogenesis, or anti-angiogenesis, is considered as a promising anticancer therapeutic strategy.

Traditional Chinese medicine (TCM) has long been recognized as a rich source for discovering drugs (Tang et al., 2003). Several anti-angiogenic components have been reported from TCM, including genistein (Fotsis et al., 1993), baicalein, baicalin (Liu et al., 2003), geniposide (Koo et al., 2004a,b), ginsenosides Rb_1 and Rg_3 (Sengupta et al., 2004; Zhang et al., 2006) and heyneanol A (Lee et al., 2006).

All these advances prompted us to examine the anti-angiogenic activities of rhubarb. In this study, zebrafish (*Danio rerio*) was used as an *in vivo* model for the detection of anti-angiogenic effects of rhubarb and its anthraquinone derivatives.

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2. Materials and methods

2.1. Materials and chemicals

Rhubarb was purchased from a herb shop in Changde, Hunan Province, PR China, in February 2008. A voucher specimen (2008-3023) was deposited in the Museum of Chinese Medicine, Institute of Chinese Medicine, The Chinese University of Hong Kong.

Endogenous alkaline phosphatase (EAP) staining was assayed with phosphatase substrate kit (Pierce, USA) and nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate, toluidine salt (NBT/BCIP) ready-to-use tablets (Roche Diagnostics GmbH, Germany). Anthraquinone derivatives were obtained from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

2.2. Preparation of ethanol extraction and fractions

The pulverized Rubarb root was extracted with 95% ethanol three times (reflux, 2 h each time), and then the ethanol extract (EE) was obtained after evaporation under reduced pressure. For solvent fractionation, EE was suspended in distilled water and extracted successively with equal volumes of *n*-hexane (Hex), ethyl acetate (EA) and *n*-butanol (BuOH), leaving a residual aqueous fraction (Aq). Each fraction was evaporated under reduced pressure to yield the extracts of Hex, EA, BuOH and Aq fractions, respectively.

2.3. Embryo handling

Zebrafish embryos were generated by natural pairwise mating as described by Westerfield (1993). The embryos were maintained in embryo water (0.2 g/L Instant Ocean® Salt, Aquarium Systems, USA) at 28.5 °C. They were manually dechorionated with forceps at 24 h post-fertilization (hpf) immediately prior to drug treatment.

2.4. Drug administration

24 hpf zebrafish embryos were arrayed in 96-well plate, one embryo per well, and incubated with 100 μl of embryo water per well containing various concentrations of an extract at 28.5 °C for 48 h. For aloe-emodin, emodin and rhein, 0.2% DMSO was used as carrier control; for chrysophanol and physcion, the mixture of 0.5% DMSO and 0.2 mM NaOH was used as carrier control.

2.5. Quantitative EAP assay on zebrafish embryo

During zebrafish development, the stage between 24 and 72 hpf has the highest angiogenic activity and quantitative EAP assay was performed as described (Parng et al., 2002). Drug-treated embryos at 72 hpf were treated by increasing concentration of ethanol for dehydration purpose. Then the embryos were washed three times with diethanolamine buffer (Pierce, USA). Next, the embryos were stained according to the protocol described in phosphatase substrate kit. After staining, 50 μl 2 M NaOH was added to stop the reaction. The optical density of soluble end product was measured at 405 nm using a microplate reader. Vessel growth was presented as percentage in optical density compared with control [% vessel formation = (OD treated day 3 − OD control day 1)/(OD control day 3 − OD control day 1) × 100%]. Each assay was repeated at least three times.

2.6. EAP staining for visual inspection

In order to inspect the blood vessels in the embryos, NBT/BCIP substrate was used to stain the blood vessels. Embryos at 24 hpf were incubated with embryo water containing PTU (final concentration 0.2 mM) before drug administration. At 72 hpf, embryos were fixed with 4% paraformaldehyde in PBST (phosphate-buffered saline + 0.1% tween-20) for 30 min. Then the embryos were dehydrated with ethanol, rinsed with PBST, and equilibrated with NTMT (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween-20). The staining reaction was started by incubating embryos with NBT/BCIP solution for about 15–30 min according to the protocol described for the NBT/BCIP ready-to-use tablets. After staining was completed, the embryos were washed with PBST.

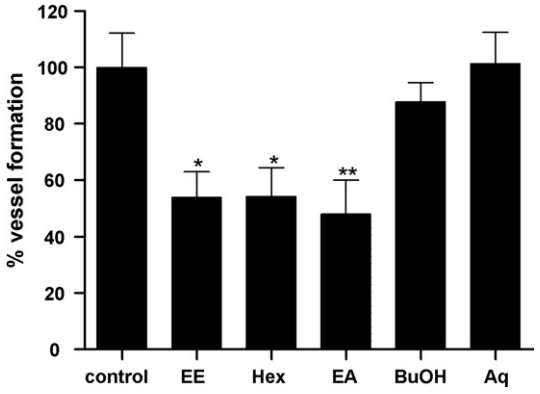

Fig. 1. Anti-angiogenic activity of the fractions of rhubarb. Each value represents the mean ± S.E.M. ($n = 10$) from a representative experiment. *Represents $P < 0.05$, **represents $P < 0.01$ in one-way ANOVA followed by the Dunnett's multiple comparison test.

2.7. Statistics

All experiments were repeated at least three times. Values are given as means ± S.E.M. Data were analyzed using Graph Pad Prism 4.0 software. Statistical significance was assessed by one-way ANOVA. *P* values less than 0.05 were considered significant.

3. Results

The 95% ethanol crude extract and Hex, EA, BuOH, and Aq fractions obtained from the 95% ethanol extract of rhubarb were examined with zebrafish angiogenic assay. As shown in Fig. 1, the ethanol extract of rhubarb inhibited vessel formation by 46% at 20 μg/ml. Successive fractionation showed that the Hex and EA fractions at 20 μg/ml potently inhibited vessel formation by 46 and 52%, respectively, in the embryos, indicating the presence of anti-angiogenic components in the two fractions. The main bioactive constituents of EA fraction are anthraquinone derivatives including aloe-emodin, chrysophanol, emodin, physicon and rhein (Fig. 2). Their existence in EA fraction was further confirmed by comparing with authentic chemical markers on TLC (figure not shown).

The anti-angiogenic activities of these anthraquinone derivatives were evaluated with zebrafish angiogenic assay. As shown in Fig. 3, three of the anthraquinone derivatives, aloe-emodin, emodin

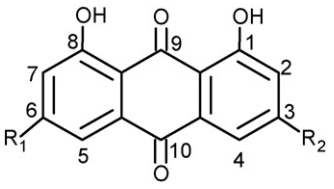

Fig. 2. Structures of the five anthraquinone derivatives tested. Aloe-emodin $R_1 = H$, $R_2 = CH_2OH$; chrysophanol $R_1 = H$, $R_2 = CH_3$; emodin $R_1 = OH$, $R_2 = CH_3$; physcion $R_1 = CH_3O$, $R_2 = CH_3$; rhein $R_1 = H$, $R_2 = COOH$.

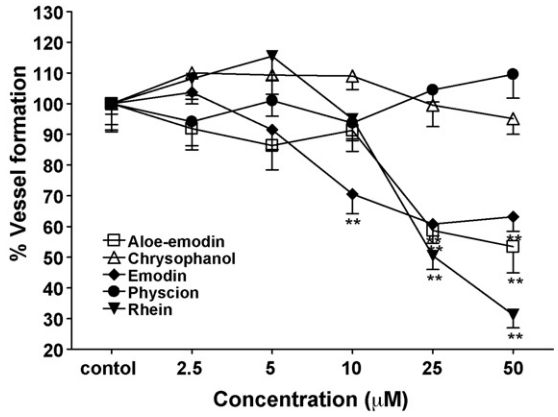

Fig. 3. Anti-angiogenic activity of anthraquinone derivatives in zebrafish model. Each value represents the mean ± S.E.M. ($n = 10$) from a representative experiment. *Represents $P < 0.05$, **represents $P < 0.01$ in one-way ANOVA followed by the Dunnett's multiple comparison test.

and rhein displayed anti-angiogenic activities in the zebrafish model. Among them, only emodin inhibited 29% of vessel formation at 10 μM, and the inhibition reached 38% at 25 μM. However both at 25 and 50 μM, rhein displayed the highest anti-angiogenic activity and inhibited 49 and 68% of vessel formation, respectively in the zebrafish model. Aloe-emodin inhibited 41 and 47% of vessel formation at 25 and 50 μM, respectively. The remaining two compounds, however, did not show any significant effect in zebrafish angiogenic model. According to the results, aloe-emodin and emodin elicited comparable responses on anti-angiogenic activities in zebrafish model to those *in vitro* and *in vivo* models (Cardenas et al., 2006; Kwak et al., 2006).

The loss of vessel formation in the embryos treated with 2.5–50 μM of rhein was further confirmed using EAP staining to visualize the vessel structures. As shown in Fig. 3, the activities of rhein showed a dose-dependent manner. The intersegmental vessels (ISVs) were the most easily observed angiogenic vessels in the embryos (Fig. 4). Treatment with 0.2% DMSO had no effect on the vessel formation and served as a vehicle control (Fig. 4A). Rhein at 25 μM partially inhibited ISV formation (Fig. 4E) and at 50 μM completely blocked ISV formation (Fig. 4F). Complete blockage of ISV formation is often associated with the appearance of pericardial edema (Figs. 4F and 5). Similar pericardial edema was found in the embryos treated by PTK787/ZK222584, which is a novel anilinophthalazine compound with a high affinity inhibitor of vascular endothelial growth factor (VEGF) receptors, and currently under phase II clinical trial for the treatment of metastatic gastrointestinal stromal tumors (Chan et al., 2002; Joensuu et al., 2008). Meanwhile, the overall morphology and structure of treated embryos were generally normal by 72 hpf. These may indicate the potential selectivity of rhein for molecule(s) involved in angiogenic signaling pathways.

4. Discussion and conclusion

Anthraquinone derivatives are the main bioactive constituents in rhubarb. Based on the position of the hydroxyl group substitution, anthraquinones are divided into two types, the emodin-type (1,8-dihydroxy-anthraquinones) and alizarin-type (1,2-dihydroxy-anthraquinones). The five compounds (Fig. 2) tested here all belong to the emodin-type. According to the results, different substitution of functional groups can affect the anti-angiogenic activity, and some structure–activity relationship (SARs) can be deduced.

Chrysophanol, emodin and physcion possess a methyl group at the C-3 position, and only differ from one another at the C-6 position. Chrysophanol with no substitution and physcion with a methoxy group substitution did not show any anti-angiogenic activity in the zebrafish model, while emodin with a hydroxyl group at C-6 position showed high activity. On the other hand, aloe-emodin, chrysophanol and rhein all have no substitution at the C-6 position, but the differences in oxidation state of the methyl group or absence of substitution at C-3 position led to dramatic differences. Among them, rhein with a carboxylic group displayed the strongest anti-angiogenic activity. Due to their planar chemical structure, the C-3 and C-6 positions of anthraquinones can be

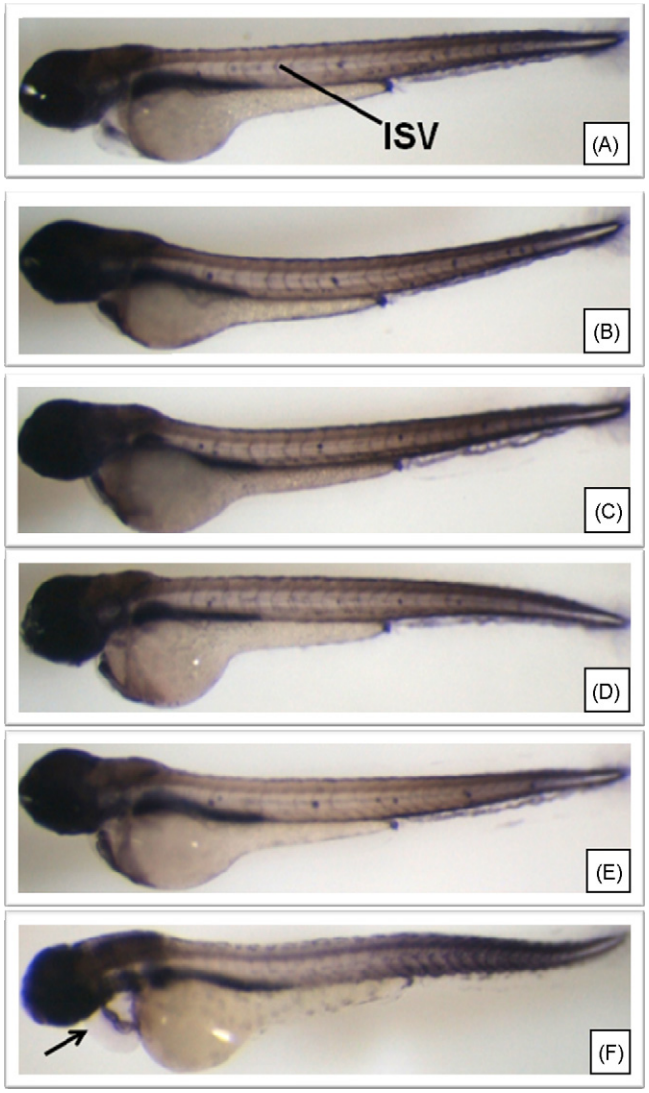

Fig. 4. Lateral view of EAP stained zebrafish embryos at 72 hpf showing embryo vessel formation after rhein treatment. (A) Control group given 0.2% DMSO; (B–F) rhein concentrations added 2.5, 5, 10, 25 and 50 μM, respectively. ISV = intersegmental vessels; the arrow on F points at pericardial edema.

Fig. 5. Lateral view of zebrafish embryos at 72 hpf showing pericardial edema (indicated by an arrow) after high-dose rhein group (50 μM) treatment.

convertible in the emodin-type. Comparing the structural characteristics of these three anthraquinones, acidic substitution with a phenolic or carboxylic group at C-3 or C-6 positions, or polar, hydrophilic substitution such as hydroxymethyl group at C-3 position may contribute to the anti-angiogenesis potency.

A few studies have also explored the anti-angiogenic potentials of aloe-emodin and emodin. The former compound could inhibit tumor cell proliferation and showed anti-angiogenic activity through inhibiting the proliferation, urokinase secretion and tubule formation of endothelial cells (Cardenas et al., 2006). Emodin, on the other hand, preferentially inhibits VEGF-A-induced angiogenesis *in vivo* and *in vitro*, possibly through blocking the phosphorylation of KDR/Flk-1 and downstream effector molecules, including FAK, ERK1/2, p38, Akt, and endothelial nitric oxide synthase (Kwak et al., 2006; Ljubimov et al., 2004; Wang et al., 2004). Rhein received much less attention, but Huang et al. (2007) reported that it could effectively inhibit the uptake of glucose in tumor cells and induce cell necrosis. Rhein also showed anti-inflammation effects through inhibiting the inducible nitric oxide synthase (Wang et al., 2002). Our findings suggested that inflammation inhibitors may have the potential to be angiogenesis inhibitors. Further search for anti-angiogenic agents should definitely include Chinese herbs used for inflammatory diseases.

Traditionally, zebrafish has been mainly used as a model organism in the fields of molecular genetics and developmental biology of vertebrates. Many zebrafish version of mammalian genes have been cloned and found to have similar functions. More recently, its value as a model organism for drug target discovery, target validation, drug discovery strategies and toxicological studies has begun to be recognized (Langheinrich, 2003; Crawford et al., 2008). Many drugs tested in zebrafish demonstrated similar effects to those observed in humans or other mammalian models. Moreover, a zebrafish angiogenic model is a recent addition to the *in vivo* models for studying anti-angiogenic agents (Taraboletti and Giavazzi, 2004). Indeed, more and more evidence show that anti-angiogenic compounds effective in mammals elicit similar effects in zebrafish (Langheinrich, 2003; Parng et al., 2002).

Unlike the traditional drug screens using cell lines or *in vitro* protein binding assays—neither of which represent the normal physiology of multi-cellular organisms, the use of zebrafish would allow the selection of bioactive compounds in a whole organism and in cells undergoing normal cell–cell and cell–matrix interactions (Stern and Zon, 2003). As compared with existing assays, the major advantages of a zebrafish-based assay are obvious: (1) hundreds of compounds can be tested simultaneously using a microplate format, (2) the assay is relatively cost-effective, fast, truly quantitative and suitable for large-scale screening, and (3) embryo maintenance, compound addition and embryo assessment are technically simple (Serbedzija et al., 1999; Zon and Peterson, 2005; Norrby, 2006). These features make zebrafish embryos an attractive model for identification of novel therapeutic agents.

However, given the heterogeneity of tissues and the molecular and cellular complexities of angiogenic reactions, it is not surprising that no single assay is optimal for all situations. There are clearly also limitations in working with the zebrafish model. First, zebrafish is an embryonic, non-mammalian model and the vasculature in zebrafish follows a similar but not entirely identical plan to that of higher vertebrates (Norrby, 2006; Serbedzija et al., 1999). Second, due to differences in molecular weight and hydrophobicity, not all small molecules are readily absorbed by zebrafish embryos, possibly leading to false negative results. Third, as zebrafish are vertebrates, country-specific animal rights legislations and institutional bioethics regulations should be included in planning experiments (Crawford et al., 2008).

Besides providing a powerful platform for drug screening, zebrafish model can also be used for probing biological processes to identify the molecular pathways, so as to generate insights into mechanisms and testable hypotheses about how a compound functions. In fact, our laboratory has worked on the anti-angiogenic components from *Tripterygium wilfordii*, a traditional Chinese folk medicine for the treatment of immune-inflammatory diseases, using zebrafish-based assays (He et al., 2009). The most potent component, triptolide, dose- and time-dependently reduced the mRNA expression of *angiopoietin* (*angpt*)*2* and *tie2* in zebrafish. Subsequent investigation of triptolide on mammalian systems including *in vitro* HUVEC assays and *in vivo* Matrigel plug and murine tumorigenesis assays showed comparable effects to those observed in zebrafish model (He et al., unpublished). All these experience further confirmed that the same angiogenesis response found in zebrafish can also be observed in mammalian models.

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