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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.

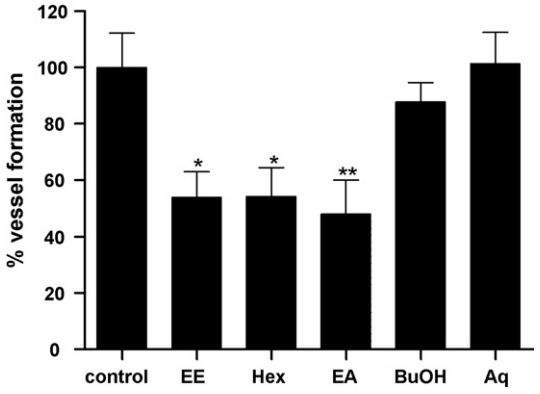

Fig. 1. Anti-angiogenic activity of the fractions of rhubarb. Each value represents the mean ± S.E.M. ($n = 10$) from a representative experiment. *Represents $P < 0.05$, **represents $P < 0.01$ in one-way ANOVA followed by the Dunnett's multiple comparison test.

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

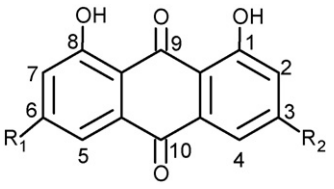

Fig. 2. Structures of the five anthraquinone derivatives tested. Aloe-emodin $R_1 = H$, $R_2 = CH_2OH$; chrysophanol $R_1 = H$, $R_2 = CH_3$; emodin $R_1 = OH$, $R_2 = CH_3$; physcion $R_1 = CH_3O$, $R_2 = CH_3$; rhein $R_1 = H$, $R_2 = COOH$.