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Anti-angiogenic activity of *Gynura segetum* leaf extracts and its fractions

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ABSTRACT

Ethnopharmacological relevance: Gynura segetum is a popular medicinal plant in Indonesia and Malaysia, known to possess various medicinal properties especially for treatment of cancer, diabetes and hypertension.
Aim of the study: This study was carried out to evaluate the anti-angiogenic effect of *Gynura segetum* leaves extracts and its fractions. The chemical compositions of the active extracts were also determined.
Materials and methods: The anti-angiogenic activity of *Gynura segetum* leaves extracts and its fractions was evaluated *in vivo* using the chick embryo chorioallantoic membrane (CAM) assay. Gas chromatography–mass spectrometry (GC–MS) analysis was carried out to identify the chemical compositions of the active extracts.
Results: The CAM treated with *Gynura segetum* leaves extracts and its fractions (100 μg/disc) showed a significantly greater anti-angiogenic effect compared to the positive control suramin (50 μg/disc). Chemical analysis of the active extracts from the leaves of *Gynura segetum* yielded nine known compounds: undecane (1), neophytadine (2), hexadecanoic acid, methyl ester (3), 9,12-octadecadienoic acid, methyl ester (4), 9,12,15-octadecatrienoic acid, methyl ester (5), phytol (6), tetradecanal (7), octadecanoic acid, methyl ester (8) and γ-sitosterol (9).
Conclusions: These results suggested that *Gynura segetum* has anti-angiogenic activity. The plant may be used as a potential source for protection against cancer.

1. Introduction

Angiogenesis, the formation of the new blood vessels from a preexisting vasculature, plays a role in pathologic processes such as the growth and metastasis of tumors (Hazel, 2003). The newly formed blood vessels can promote cancer growth by supplying nutrients and oxygen and by removing waste products. Metastasis also depends on angiogenesis, as tumor cells are shed from a primary tumor and grow at their target organs (Miyazawa et al., 2009). Thus, the inhibition of angiogenesis, or anti-angiogenic activity, is considered as a promising approach for treatment of cancer (Mathur et al., 2006; He et al., 2009b).

Increasing interest in anti-angiogenic therapy for cancer requires the development of a quantitative angiogenesis assay (Adini et al., 2009). According to Hazel (2003), one useful *in vivo* system that has been used extensively in angiogenesis research is the highly vascularized chick embryo chorioallantoic membrane of the chicken embryo. Chick embryo chorioallantoic membrane (CAM) has become a widely used tool for the determination of both angiogenesis and anti-angiogenic activities of many drugs including herbal extracts (Peng et al., 2009). The success of anti-angiogenic therapy for cancer treatment has led to an explosion in the research for anti-angiogenic agents (He et al., 2009a).

Traditional herbal medicines have long been recognized as a rich source for discovering such agents. Up to the present, many herbs and phytochemicals have been shown to have anti-angiogenic activities both *in vitro* and *in vivo*, e.g. *Benincasa hispida* (Lee et al., 2005), *Withania somnifera* root (Mathur et al., 2006), *Ulmus davidiana* var. *japonica* (Jung et al., 2007), *Saururus chinensis* (Yoo et al., 2008), *Gastrodia elata Blume* (Ahn et al., 2007) and *Tripterygium wilfordii* (He et al., 2009a).

Gynura segetum (daun dewa in Malay), which belongs to family Compositae is a well recognized medicinal plant in Indonesia and Malaysia. The plant was claimed to possess various medicinal values including possible treatment of cancer, diabetes and hypertension (Suharmiati and Maryani, 2003).

To the best of our knowledge, there is no other study reported on the *in vivo* anti-angiogenic activity of *Gynura segetum* leaves. Therefore, in present study *in vivo* anti-angiogenic

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effect of *Gynura segetum* leaf extracts and fractions were evaluated using the CAM assay. The chemical composition of the potential anti-angiogenic constituents in active extracts was identified by Gas chromatography–mass spectrometry (GC–MS) analysis.

2. Materials and methods

2.1. Chemicals and reagents

All the chemicals and solvents of analytical grades included petroleum ether (60–80 °C), methanol, chloroform, acetone, ethyl acetate, *n*-butanol, and ethanol 95% were purchased from Merck (Darmstadt, Germany). Suramin was purchased from Sigma Chemicals (St. Louis, MO, USA).

2.2. Plant material

The leaves of the plants were collected from Jabatan Pertanian Relau, Penang and identified by Mr. Adenan Jaafar, a taxonomist from the School of Biological Sciences, University Sains Malaysia. A voucher sample of the plant, reference number 11013 was deposited at the herbarium of School of Biological Sciences, University Sains Malaysia.

2.3. Preparation of leaves extracts and fractions

Freshly collected leaves were dried in an oven at about 45 °C and grounded to fine powder. The powdered leaves (700 g) were first extracted with petroleum ether (60–80 °C) for 3 days, followed by chloroform and finally with methanol using Soxhlet apparatus. The extracts were evaporated in a rotary evaporator at a maximum temperature of 45 °C to yield 93.03 g (13.29%) of petroleum ether extract, 107.38 g (15.34%) of chloroform extract and 150 g (21.48%) of methanol extract.

Methanolic extract (30 g) was dissolved in 300 ml hot distilled water and then partitioned successively with equal volumes (300 ml 3×) of chloroform ($CHCL_3$), ethyl acetate (EA), and *n*-butanol (BuOH) by liquid–liquid extraction method. All these partitions were separately concentrated under reduced pressure to yield 2.91 g (9.71%) of $CHCL_3$ fraction, 9.45 g (31.5%) of EA fraction, 5.82 g (19.4%) of BuOH fraction.

2.4. Preparation of test sample

The test samples were dissolved in ethanol (95%) to a final concentration of 10 mg/ml. For the preparation of the discs, 10 μl of these solutions were applied dropwise on sterile filter paper of 3 mm diameter, dried under a laminar flow hood to give concentration of 100 μg per disc. Suramin at a concentration of 50 μg per disc was applied as positive control and CAMs treated with equal volume of ethanol as blank control.

2.5. Chick embryo chorioallantoic membrane (CAM) assay

The CAM assay was performed according to the method given by West et al. (2001) with some modifications. Fertilized chicken eggs were incubated in an incubator (Incucell, Germany) for 3 days at 37 °C under a constant relative humidity of 80%. The eggs were positioned in a horizontal position and rotated several times in a day. On day-3, eggs were swabbed with 70% alcohol under a laminar flow hood. Albumin (2–3 ml) was withdrawn using a syringe with a 21-gauge needle through the pointer end of the egg in order to allow detachment of the developing CAM from the eggshell. A window (Fig. 1A) was then cut in the shell using a fine-cutting tool, and the shell was then removed with sterile forceps. This window served as a portal of access for the CAM. Any nonviable eggs were disposed at this stage, and for each of the remainder, the window was closed with a cellophane tape. The eggs were returned to the incubator, keeping them horizontally with the window uppermost, until sample application on day 5.

On day-5, photograph of the embryos were captured with the use of digital camera (Sony Cyber-shot DSC-T33) to show blood vessels where the disc was going to be placed above it. Filter paper discs with the test substances were placed directly using microsurgical forceps over a blood vessel on the growing CAM at day-5 incubation under sterile conditions (Fig. 1B and C). A second photograph was taken to obtain the image of the discs position on the CAM. The eggs were closed with the cellophone tape and returned to the incubator. The final evaluations were carried out on day-7 of development. On day-7, the filter paper disc was gently discard from CAM and examined for anti-angiogenic effect at site of sample application. A third photograph was taken to obtain the image of the CAM after treatment with various extracts or standard drug.

Images of the surface of the same CAM within the same test sample (before and after treatment) were compared and the anti-angiogenic effect will be quantified solely in the area of the CAM covered by the filter paper disc. A modified semi quantitative score system (Bűrgermeister et al., 2002; Krenn and Paper, 2009) with a scale of 0–2 was used for grading (see Table 1). The degree of anti-angiogenic effect was recorded blindly by two independent observers.

Eighteen eggs per test sample were prepared to allow for the 30–40% mortality inherent to the procedure and to yield a minimum of 8–10 available eggs per test sample. For every test sample, the average score was calculated and the interpretation of anti-angiogenic effect was quantified as follows:

Average score < 0.5 = no anti-angiogenic effect (inactive).
0.5 ≤ average score ≤ 1 = weak anti-angiogenic effect.
1 < average score < 1.5 = good anti-angiogenic effect.
Average score ≥ 1.5 = strong anti-angiogenic effect.

2.6. Statistical analysis

The data were expressed as means ± S.D. Statistical significances were analyzed using ANOVA followed by Turkey's multiple range tests. The statistical analysis was performed using SPSS for Windows (SPSS, Inc.). *P* values less than 0.05 was considered to be significant.

2.7. Gas chromatography–mass spectrometry (GC–MS) analysis

The GC–MS analysis of the active extracts (chloroform extract and chloroform fraction) were performed using an Agilent 6890 gas chromatography instrument coupled to an Agilent 5973 mass spectrometer and Agilent ChemStation software (Agilent Technologies, Palo Alto, CA, USA). Compounds were separated on a HP-5MS, 30 m × 0.25 mm i.d. capillary column coated with 0.25 μm film. The oven temperature was 70 °C which was held for 2 min, followed by an increase in temperature to 280 °C and this was held isothermally for 20 min. Helium was used as carrier gas of 1.2 ml min^{-1} flow rate. The injector and detector temperature were 250 °C and 280 °C, respectively. The parameters of HP 5973 mass detector were: ion mass/charge ratio, 20–500 *m*/*z*; scan mode and the ionization energy were 70 eV.