Comparative Study of Anti-oxidative Effects of Tibetan Folk Medicine *Erigeron multiradiatus* during Plant Growth

ZHANG Zhi-feng[1*], LIU Yuan[1], LUO Pei [2], ZHANG Hao[3]

1. Ethnic Pharmaceutical Institute of Southwest University for Nationalities, Chengdu 610041, China
2. Teaching Division, School of Chinese Medicine, Hong Kong Baptist University, Kowloon Tong, Kowloon, Hong Kong, China
3. West China School of Pharmacy, Sichuan University, Chengdu 610041, China

Abstract: **Objective** To explore the effects of a potential anti-oxidative plant, *Erigeron multiradiatus* (Asteraceae), plant materials from naturally distributed high-altitude populations at different stages of life cycle were collected. **Methods** Fifteen extracts obtained from the Ganzi region (Sichuan, China) were studied to assess their radical-scavenging ability on 1,1-diphenyl-2-picrylhydrazyl radicals and reducing power ability. Moreover, considering that anti-oxidants and free radical scavengers can also exert protective effect on endothelial cells from oxidative injury, these extracts were also evaluated for their anti-oxidative activity against cellular injury in the cultured human endothelial cell line (ECV304) induced by hydrogen peroxide (H_2O_2). **Results** All the extracts had radical-scavenging and/or reducing power ability, and the most active extract was found during flowering whereas the lowest appeared during vegetative growth period. The accumulation of anti-oxidative compounds was found to be affected by the altitude of growth environment. Total flavonoid content assay was also performed to support this outcome. Furthermore, these extracts also exhibited different effects on attenuating H_2O_2-induced cytotoxicity and inhibiting lipid peroxidation and LDH leakage from endothelial cells. **Conclusion** *E. multiradiatus* may be an important natural anti-oxidant and this property may contribute to verifying the utilization of this plant in Tibet folk medicine.

Key words: anti-oxidative activity; developmental stage; endothelial cells; *Erigeron multiradiatus*; hydrogen peroxide

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Introduction

Erigeron multiradiatus (Lindl.) Benth, a heat clearing and detoxicating folk herb, belongs to the genus *Erigeron* L. (Asteraceae). This plant has been used in traditional Tibetan medicine for years to treat various diseases related with inflammation, such as rheumatism, hemiparalysis, hyperpiesia, hepatitis, adenolymphitis, and enteronitis (Yang, 1991). *E. multiradiatus* contains a notable amount of flavonoids represented by scutellarin and apigenin, reported by Zhang *et al* (1998). Five active compounds in *E. multiradiatus* were also determined simultaneously (Zhang *et al*, 2008). Furthermore, as a part of our investigation project in the evaluation of biological activities related to ethnopharmacological uses of the plants in *Erigeron* L., the anti-inflammation through bioassay-guided procedure was also demonstrated in previous studies (Luo *et al*, 2008; Zhang *et al*, 2009). Oxidative stress plays a key role in the pathophysiologic process of acute and chronic inflammatory diseases. Intracellular components such as lipids and nucleic acids are easily and rapidly oxidized by excessive reactive oxygen species (ROS), and such reactions lead to cell damage. Moreover, *E. breviscapus* (vant.) Hand-Mazz., a species with the same vernacular name "*Meiduoluomi*" as *E. multiradiatus*, recently has been shown to have anti-oxidative activity (Liu *et al*, 2005), which promoted us to study the anti-oxidative properties of *E. multiradiatus*.

Oxidative stress is defined as an imbalance between local ROS production and anti-oxidative mechanisms

* Corresponding author: Zhang ZF Tel: +86-28-8552 2315 E-mail: zhangzhf99@gmail.com
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(Kimura *et al*, 2005). The harmful action of ROS can be blocked by anti-oxidative substance (Sies, 1997; Galli *et al*, 2005). Although several synthetic anti-oxidants have been used, their various side effects and toxicities have become an issue. Therefore, natural anti-oxidants have attracted much attention and great efforts have been made to search for safe and effective therapeutic agents for the treatment of oxidative stress-related diseases. The accumulation of the anti-oxidant substance in plant is generally regulated by genetic factors, and also strongly influenced by biotic and abiotic stimuli, such as pathogens and light conditions (Dixon and Paiva, 1995). Great differences in anti-oxidative properties have also been observed among the different parts of a plant in one species depending on environmental conditions and the harvest (Judzentiene, Stikliene, and Kupcinskiene, 2009). Considering that many of above factors associated with anti-oxidant accumulation, evaluation of wild cultured medicinal plants in relation to anti-oxidative activity could offer better understanding of traditional collection guidance and provide evidence to the likely location of plant high in anti-oxidative activity. *E. multiradiatus* is mainly distributed in alpine and subalpine meadow of Qinghai-Tibetan Plateau, at the altitude 2600－4300 m. The parts used traditionally as medicines were the whole plant, such as leaves, petioles, flowers, and capsules. In local area, gathering practices generally took place during late spring and summer. Traditional collection practice and the viability of plant growth conditions were related to the use of *E. multiradiatus* as a potential anti-oxidant. Therefore, it was important to characterize the variability of anti-oxidative effects of *E. multiradiatus* at different growth stages. This evaluation was undertaken to determine whether the accumulation of anti-oxidative compounds was affected by growth environment or harvest.

In the present study, selection, collection, and preparation of 15 sets of *E. multiradiatus* samples in the Ganzi region, Qinghai Tibetan Plateau area of West China were performed to compare their radical-scavenging and reducing power. Moreover, considering that anti-oxidative effects can exhibit a direct or indirect influence with endothelial cellular damage, these samples were also evaluated for their protective effects against intracellular oxidative stress stimulated by hydrogen peroxide (H_2O_2) in endothelial cells (ECV304) which, to the best of our knowledge, has not been reported for *E. multiradiatus* so far.

Materials and methods

Chemistry reagents

Rutin was purchased from the National Institute for Food and Drug Control (Beijing, China). 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 3-[4,5-dimethylthiazol-2-yl]-2,5-dephenyl tetrazolium bromide (MTT), and dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and other cell culture materials were purchased from Gibco-BRL (USA). MDA and LDH Assay Kits were from Jiancheng Bio-engineering Institute (Nanjing, China). All other reagents are of analytical grade (Guangdong, China).

Plant materials collection

Fifteen sets of *E. multiradiatus* samples studied in this work were shown in Table 1, and some information was mentioned about their places of collection, dates of collection, and parts for medicinal use. All the samples were obtained from five selected locations on the eastern Qinghai-Tibetan Plateau of China. The harvest was between May and September, 2007. During the vegetative stage (May, 2007), only the top aerial parts of the plant were collected, including leaves and petioles. During flowering stage (July, 2007), leaves and petioles, as well as flowers were harvested. During fructification stage (September, 2007), only those plants which have already shown capsules developed were selected. The samples of *Erigeron multiradiatus* (Lindl.) Benth were authenticated by Prof. ZHANG Hao in the West China School of Pharmacy, Sichuan University. Voucher specimens were deposited in the Herbarium Center of West School of Pharmacy, Sichuan University, China. The samples and geographical harvesting locations were shown in Figs. 1 and 2.

Preparation of extracts

The plants were dried at room temperature and crushed into powder, respectively. A portion of each sample (25 g) was extracted by reflux with 200 mL methanol for 2 h at 60 ℃. Then the sample extraction procedure was repeated twice and the supernatants were combined together. The methanol extract of *E. multiradiatus*

A **B** **C**

Fig. 1 Plant pictures of *E. multiradiatus* in vegetative (A), flowering (B), and fructification (C) stages

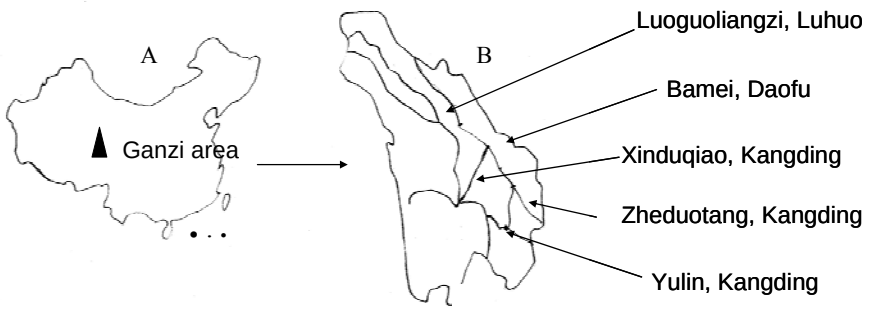

Fig. 2 Geographical location of field research areas in Ganzi Tibetan Autonomous Prefecture, Sichuan Province, China
A: Map of China B: Map of Ganzi, Sichuan Province

(MEE) was filtered with 4.5 μm filters and concentrated up to dryness under vacuum and stored in a brown glass bottle at 4 ℃ for following experiments.

Determination of total flavonoids

Total flavonoid contents were measured according to colorimetric assay with small modification (Jia, Tang, and Wu, 1999). A Shimadzu 1601 UV-vis spectrophotometer with a pair of matched quartz cuvettes (Hellma) was used for absorbance measurements. Aliquot (1 mL) of rutin standard solution at different concentrations or appropriately diluted samples was added into a 10 mL volumetric flask containing 4 mL of deionized H_2O. At initial time, 0.3 mL of 50 mg/mL $NaNO_2$ was added into the flask. After 5 min, 0.3 mL of 100 mg/mL $AlCl_3$ was added. At 6 min, 2 mL of 40 mg/mL NaOH was added into the mixture. Immediately, the solution was diluted to 10 mL with deionized H_2O and mixed thoroughly. The absorbance was measured at 510 nm after standing for 15 min. The content of total flavonoids was expressed as rutin equivalents by reference to the rutin standard calibration curve. Samples were analyzed in three replications.

DPPH scavenging effect

The abilities of MEE to scavenge DPPH radicals were evaluated by using the method of Shimada *et al* (1922) with a little modification. Each extract of different concentrations in 4 mL methanol was mixed with 1 mL of methanol containing DPPH radicals, a final concentration of 0.2 mmol/L DPPH was gained. After gentle mixing and standing for 30 min at 25 ℃, the absorbance value was read against a blank at 517 nm with a microplate reader. The anti- oxidative activities of the samples were expressed by the inhibitory rates of DPPH radicals. The DPPH solution without extract was used as control sample. All the tests were carried out in triplicate.

Reducing power determination

According to the method of Oyaizu (1986) with small modification, the reducing powers of MEE were determined. Different amounts of the extracts were mixed with sodium phosphate buffer (2.5 mL, 200 mmol/L, pH 6.6) and potassium ferricyanide $[K_3Fe(CN)_6]$ (2.5 mL, 10 mg/mL). The mixture was incubated for 20 min at 50 ℃. A portion (2.5 mL) of trichloroacetic acid (100 mg/mL) was added into the mixture and centrifuged for 10 min. The upper layer of the solution (2.5 mL) was mixed with deionized H_2O (2.5 mL) and $FeCl_3$ (0.5 mL, 1 mg/mL), and the absor-

bance value was measured at 700 nm. The absorbance value increased in the reaction mixture, which was represented as the reducing power was also increased.

Determination of anti-oxidative activity in cultured ECV304 cells

To further investigate the anti-oxidative effects of the methanol extracting of *E. multiradiatus* harvested during flowering stage assay was performed.

A spontaneously-transformed line derived from the human umbilical vein endothelial cells, ECV304 cell line, was purchased from China Center for Type Culture Collection (CCTCC), which was incubated at 37 ℃ in a humidified atmosphere of 5% CO_2 on culture plates with 10% fetal bovine serum-supplemented DMEM medium. Subcultures were also used between the passages 4 to 6 for all experiments.

The H_2O_2-induced cellular oxidative model was used to assay the protection against oxidative damage. The incubated time and the appropriate concentration of H_2O_2 were determined in a preliminary experiment. After cell confluence had been taken, MEE was firstly treated in the tested wells at a final concentration of 50 μg/mL. Then the plates were incubated under routine conditions for 24 h. The medium was changed and cells were exposed to H_2O_2, which was freshly prepared for another 4 h except normal control.

The MTT assessment was performed for cell viability, which was referenced by Mosmann *et al* (1983). A volume of 200 μL 0.5 mg/mL MTT was added into each well. After the resultant formazan crystals were dissolved in 150 μL of DMSO, the absorbance value was read at 570 nm with a microplate reader in each well (Bio-Rad 3550). However, the cell viability of the control group, which was not exposed to H_2O_2, was defined as 100%. And the numbers of surviving cells in the treated groups were expressed as the percent of the control group. The results were given as the average value for the triplicate determinations.

Cell damage was also evaluated by determination of lactic dehydrogenase (LDH) release from the cell supernatant and malondialdehyde (MDA) in the cell lysate. The contents of MDA and LDH were measured using assay kits respectively, according to the manufacturer's instructions.

Statistical analysis

Data were expressed as $\bar{x} \pm s$ deviation. Statistical analysis of data in each group was carried out using One-way ANOVA and Duncan's multiple range test with SPSS11.5 software, and $P < 0.05$ was considered statistically significant difference among groups.

Results and discussion

Extract yield and total flavonoid content

The last two columns of Table 1 showed the extract yields and total flavonoid contents of MEE from different samples during plant growth. Referring to the different collecting places, the samples of Bamei had the highest yield (17.9%) that was observed during the flowering stage. Moreover, we observed that the yields of different medicinal parts in plant have no significant variation.

Total flavonoid contents of MEE in the different parts of medicinal plant collected respectively in Yulin, Zheduotang, Xinduqiao, Bamei, and Luoguoliangzi did not vary significantly and ranged from 30.8 to 56.8 mg, using rutin as equivalent in 1 g of MEE. Except for Luoguoliangzi, flavonoid was found in higher concentration of the collection place with higher altitude, which revealed that the biosynthesis of flavonoid was affected to some extent by growth environment. However, among extracts of the plant from the same collection place, the differences of their flavonoid contents were strongly depending on the collection date of the plants with a peak at the flowering stage (July, 2007). It was obvious that more flavonoid components were accumulated during flowering stage than at the beginning of fructification (September, 2007) during which their contents decreased rapidly, whereas the lowest level was found in the vegetative stage (May, 2007). This variation can be expected for plant extracts, due to the developmental stages of the plants and distribution of flavonoids components.

Radical-scavenging activity

The results of the free radical-scavenging activity of the different extracts are shown in Fig. 3. DPPH is a stable free radical used as a reagent to evaluate free radical-scavenging activity (Piao *et al*, 2006). The effect of anti-oxidants on DPPH radical-scavenging was considered due to their hydrogen-donating ability. Different samples showed varied radical-scavenging effects on DPPH. With the samples from Xinduqiao,

Table 1 Sample number, collection information (site, date, and altitude), plant part used, methanol extract yield, and total flavonoid content of *E. multiradiatus*

Sample No.	Collection site	Collection month	Plant parts used	Growing altitude / m	Yield / %[a]	Total flavonoid[b]/mg
YLKD-1	Yulin, Kangding	May, 2007	leaves, petioles	2600	15.8	31.2 ± 4.2
YLKD-2	Yulin, Kangding	July, 2007	flower, leaves, and petioles	2600	16.2	45.4 ± 7.5
YLKD-3	Yulin, Kangding	Sep., 2007	capsules, leaves, and petioles	2600	15.8	38.4 ± 1.3
ZDTKD-1	Zheduotang, Kangding	May, 2007	leaves, petioles	3000	16.4	32.4 ± 3.6
ZDTKD-2	Zheduotang, Kangding	July, 2007	flower, leaves, and petioles	3000	16.3	49.6 ± 4.4
ZDTKD-3	Zheduotang, Kangding	Sep., 2007	capsules, leaves, and petioles	3000	16.9	35.9 ± 5.1
XDQKD-1	Xinduqiao, Kangding	May, 2007	leaves, petioles	3500	17.2	37.3 ± 2.5
XDQKD-2	Xinduqiao, Kangding	July, 2007	flower, leaves, and petioles	3500	16.8	50.5 ± 4.7
XDQKD-3	Xinduqiao, Kangding	Sep., 2007	capsules, leaves, and petioles	3500	17.4	39.2 ± 4.9
BMDF-1	Bamei, Daofu	May, 2007	leaves, petioles	3600	17.2	40.3 ± 3.4
BMDF-2	Bamei, Daofu	July, 2007	flower, leaves, and petioles	3600	17.9	56.8 ± 4.5
BMDF-3	Bamei, Daofu	Sep., 2007	capsules, leaves, and petioles	3600	17.6	42.3 ± 3.2
LGLZLH-1	Luoguoliangzi, Luhuo	May, 2007	leaves, petioles	4300	17.5	30.8 ± 5.3
LGLZLH-2	Luoguoliangzi, Luhuo	July, 2007	flower, leaves, and petioles	4300	17.2	44.3 ± 2.6
LGLZLH-3	Luoguoliangzi, Luhuo	Sep., 2007	capsules, leaves, and petioles	4300	17.6	37.5 ± 6.1

a: Extract yields expressed as milligrams of extract per gram of the used plant parts (dry weight)

b: Total flavonoids content was expressed as rutin equivalent: milligrams of rutin per gram of the used plant parts (dry weight). Each value is expressed as $\bar{x} \pm s$ (n = 3)

Kangding, and Bamei, Daofu extracts at flowering stage possessing, the best free radical-scavenging activity was observed (IC_{50} = 93.9 and 91.5 μg/mL, respectively). The lowest radical-scavenging activity was exhibited by the sample from Xinduqiao, Kangding extract at vegetative stage (IC_{50} = 367.1 μg/mL). The differences of radical-scavenging activity in the samples from five places in Ganzi may account in part for the variable flavonoid contents in these samples. But this could not explain why such a little variation of flavonoids led to so much difference in the activity. We supposed that flavonoids could exhibit the anti-oxidative effect of *E. multiradiatus* but other potential active components also contributed to the action. Therefore, only assays of flavonoid content and some chemical reaction were restricted in the evaluation of the anti-oxidative properties of plant extract. Some *in vitro* cell screening methods, for instance, endothelial cell injury by artificial oxygenant, may be needed to confirm the assessment results in anti-oxidative potency.

Further, the extracts also showed a moderate reductive capability. We investigated the transformation of Fe^{3+} to Fe^{2+} in the presence of *E. multiradiatus*. Yen and Duh (1983) concluded that a direct correlation existed between anti-oxidative activities and reducing power of the plant extract. MEE significantly exhibited anti-oxidative action by breaking the free radical chain. As shown in Fig. 4, our study on the reducing power of all the tested extracts suggested that it was also likely to contribute significantly towards the observed anti-oxidative effect.

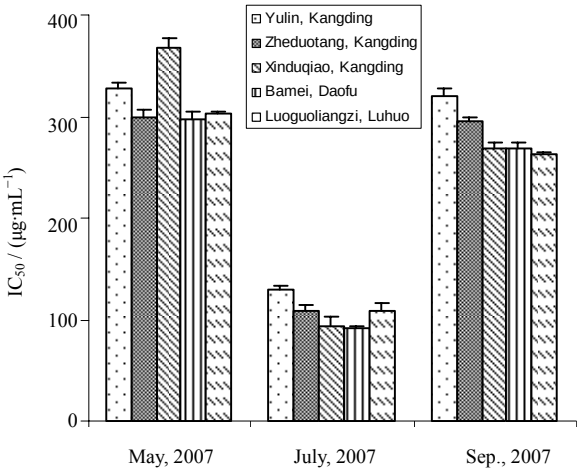

Fig. 3 Comparison on DPPH scavenging effect of MEE from different collection months and sites ($\bar{x} \pm s$, n = 3)

According to the study of anti-oxidative activity

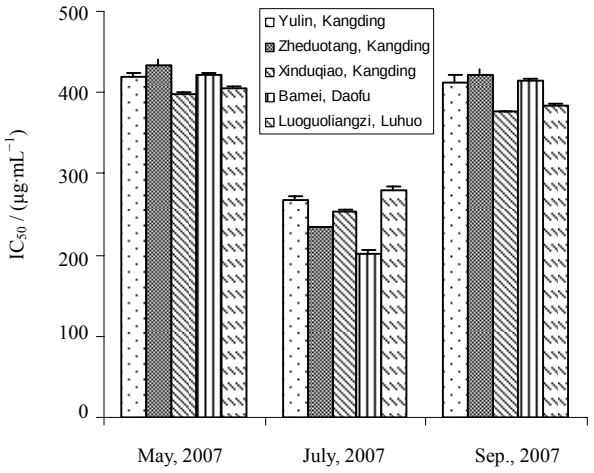

Fig. 4 Comparison on reducing power of MEE from different collection dates and sites ($\bar{x}$ ± *s*, *n* = 3)

in vitro, the DPPH radical-scavenging activity and reducing power of all extracts showed the increasing trend with the increasing concentration of flavonoid components of the plant extracts. At different growth phases of this species, the anti-oxidative activity was arranged in following order: flowering > fructification > vegetative. A dose-response relationship was found in the DPPH radical-scavenging activity and reducing power; The activity was increased as the concentration of flavonoids increased for each individual sample.

Protection against H_2O_2-induced endothelial cellular damage

Results in Table 2 suggested that the cell viability evaluated by MTT was significantly decreased after ECV304 cells exposed to H_2O_2. Vitamin E (Vit E), a potential anti-oxidant, at 5 μg/mL was also significantly inhibited as cytotoxicity was induced by H_2O_2. MEE exhibited a potent protection against H_2O_2-induced ECV304 cytotoxicity in culture as well as Vit E did. Pretreating these cells with MEE for 24 h markedly suppressed the damage of H_2O_2 in a dose-dependent manner and significant effects were observed at the highest concentration (100 μg/mL).

LDH and MDA assessments, other indicators of cell toxicity, were performed. The lipid bilayer plasma membrane was considered as an important target of free radicals. Their peroxidation may increase permeability, impair membrane functions, and inactivate membrane-bound enzymes and receptors (Bast, Haenen, and Doelman, 1991). When the membrane was damaged, both LDH and MDA will be rapidly released into the

Table 2 Comparison on protection of MEE from different collection samples on H_2O_2-induced cellular injury ($\bar{x}$ ± *s*, *n* = 3)

Groups	Cell viability / %	MDA / (μmol·g^{-1})	LDH / (kU·mL^{-1})
YLKD-1	39.8 ± 4.5	13.60 ± 0.58	54.97 ± 5.68
YLKD-2	55.2 ± 3.5*	8.90 ± 0.46**	39.67 ± 5.46**
YLKD-3	45.7 ± 5.7	14.52 ± 0.56	57.15 ± 6.13
ZDTKD-1	40.5 ± 5.7	12.45 ± 0.67	55.48 ± 7.98
ZDTKD-2	54.3 ± 11.3**	9.02 ± 0.56**	41.69 ± 6.25**
ZDTKD-3	47.8 ± 6.4	14.70 ± 0.46	58.13 ± 7.59
XDQKD-1	42.4 ± 2.9	12.56 ± 0.23	64.35 ± 6.45
XDQKD-2	55.8 ± 4.7*	9.45 ± 0.73**	42.58 ± 8.46**
XDQKD-3	40.4 ± 7.9	13.75 ± 0.78	60.12 ± 7.12
BMDF-1	49.5 ± 6.7	12.20 ± 0.32	61.25 ± 8.13
BMDF-2	61.1 ± 5.2**	8.56 ± 0.34**	38.59 ± 6.37**
BMDF-3	41.5 ± 3.9	14.25 ± 0.88	58.26 ± 8.24
LGLZLH-1	46.3 ± 5.2	12.43 ± 0.51	62.15 ± 4.69
LGLZLH-2	58.1 ± 4.9**	8.78 ± 0.23**	40.29 ± 5.11**
LGLZLH-3	44.8 ± 6.9	12.48 ± 0.45	57.36 ± 9.76
Control	100 ± 3.9	4.23 ± 0.58	19.05 ± 1.98
H_2O_2	42.6 ± 5.3$^{\#\#}$	13.98 ± 0.42$^{\#\#}$	67.97 ± 7.61$^{\#\#}$
H_2O_2 + Vit E	62.4 ± 4.9**	7.32 ± 0.37**	28.43 ± 3.58**

$^{\#\#}P < 0.01$ *vs* control group; $^{*}P < 0.05$ $^{**}P < 0.01$ *vs* H_2O_2 group

cell culture supernatant and cause an increase in LDH and MDA in the culture supernatant. The present studies have clearly shown that the exposure of ECV304 cells to H_2O_2 for 4 h rapidly increased lipid peroxidation as measured MDA and LDH and significant attenuation of cell viability. As shown in Table 2, the H_2O_2-induced cell death was ameliorated by MEE. When ECV304 cells were pre-incubated with 10, 50, and 100 μg/mL MEE or Vit E prior to H_2O_2 treatment, a significant reduction in MDA and LDH release was obtained, as comparing with H_2O_2 control cells. The results demonstrated that the protective effect of MEE on H_2O_2-induced cytotoxicity measured by LDH and MDA release was similar with that determined by MTT assay. The mechanism may involve direct scavenging radicals, chelating the transition metal ions, such as Fe^{3+} and Cu^{2+}, which prevent the formation of OH^{-} from H_2O_2 via the Fenton reaction, and inhibiting the lipo-oxygenase (Bors *et al*, 1990).

Our prescreening results suggested that the anti-oxidative activity of *E. multiradiatus* might be likely due to flavonoids, which allowed this plant to be considered as a potential source of anti-oxidant. The total flavnoid content and the anti-oxidative activity of *E. multiradiatus* had some correlation. Therefore, flavonoids are proposed

to be the potential candidates of anti-oxidants from *E. multiradiatus*. In this study, the anti-oxidative activities of MEE varied with used parts of plant during developmental stages in test models. Results revealed that the aerial parts of *E. multiradiatus* from flowering stage showed the strongest anti-oxidative activities. On the other hand the underground parts, with low flavonoids content possessed the least anti-oxidative activity. The properties of extracting plant part significantly affected the anti-oxidative activity of the extracts. From the present results, extracts in aerial parts had higher anti-oxidative activity than those in under-ground parts, possibly because major anti-oxidants are more accumulative in flowers and leaves than in roots. Thus flowers and leaves of *E. multiradiatus* are the good medicinal materials to extract the active compounds for anti-oxidative treatment. In addition, we suggest developing the usage in the aerial parts of the plant to prevent the over-exploitation of *E. multiradiatus*.

Till now there are various methods to investigate the anti-oxidative properties of plant extracts and compounds. And the differences of the anti-oxidative activity of the extracts possibly vary with the evaluation approaches. Therefore, our present study makes two chemical reaction methods combined with cellular level measurements used due to the differences between the analytic methodologies. Our results obtained from this study correlate the anti-oxidative activity with the flavonoid components in *E. multiradiatus* and initially explain the possible beneficial effect of *E. multiradiatus* against endothelial cells following the damage of H_2O_2 *in vitro*. Furthermore, these data also support the use of *E. multiradiatus* in traditional Tibetan medicine and suggest the potential development in anti-oxidative therapy.

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