Antiproliferative Effect of *Eupatorium riparium* Reg. Leaves Benzene Extract Against C2C12 and MKN45 Cell line In Vitro

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Abstract

Eupatorium riparium *Reg. belongs to Asteraceae family. Cytotoxic activity of* E. riparium *leaves has been demonstrated to have an in vitro cytotoxic effect on cancer cell lines. The aim of this research was to know antiproliferative activity of benzene extract of* E. riparium *leaves against C2C12 (adherent mouse myoblast cell line) and MKN45 (gastric cell line). Leaves powder was extracted using benzene. Antiproliferative effect was determined by Cell Proliferation Reagents WST-1 Test for 1h, 2h, and 4h after incubation for 72h. The result showed that benzene extract of* E. riparium *leaves possessed remarkable antiproliferative effect against C2C12 cell line with IC_{50} values of 119.14µg/ml (1h), 100.74 µg/ml (2h) and 70.65 µg/ml (4h), and MKN45 cell line with IC_{50} values of 201.74µg/ml (1h), 135.06 µg/ml (2h) and 124.54µg/ml (4h) respectively. The result indicated that benzene extract of* E. riparium *leaves possess potentials antiproliferative against C2C12 and MKN45 cell. Further study is necessary to know the anticancer mechanisme on C2C12 and MKN45 cell line.*

***Key words**:* Eupatorium riparium *Reg, Antiproliferative, C2C12, MKN45, WST-1*

INTRODUCTION

Plants produce a diverse range of bioactive molecules, making them rich sources of different types of medicines. Most of the drugs today are obtained from natural sources or semi synthetic derivatives of natural products used in the traditional systems of medicine. Thus it is a logical approach in drug discovery to screen traditional natural products.

A large number of the plants are claimed to possessed the antimalarial, anticancer, antimicrobial, antifungal, anti-inflammatory, antibiotic properties in the traditional system and they are also used extensively by the tribal people worldwide (Ananil *et al.,* 2000; Rahmatulla *et al.,* 2010; Aranda *et al.,* 2011).

The genus Eupatorium belongs to the Eupatorieae, one of the 13 tribes of the Asteraceae and comprises of nearly 1.200 species distribute mainly in tropical region of America, Europe, Africa and Asia (Zhang *et al.,* 2008; Chakraborty *et al.,* 2011).

In Java, Indonesia, the genus Eupatorium is represented by approximately seven species, one of these species is *Eupatorium riparium* Reg. This Plant is spreading subshrubs from a creeping rootstock, stems sprawling, 40-60 cm long, sparsely puberulent. The numerous stems tend to spread horizontally at first, rooting at the joints where they contact the soil. The leaves are lanceolate to elliptic in shape, 4-8 cm long, 1-1.5 cm wide, having margins sharply serrate and petioles 0.5-

1.3 cm long. Inflorescences lax, involucral bracts 4-5 mm long, puberulent, corollas white, 3-3.5 mm long. Achenes black, 1.5-2 mm long, puberulent on the angles. This plant grows in humid tropical or subtropical rainforests, favours shaded riverbanks or steep, south-facing hill sides, and other sheltered moist places and a troublesome and invasive weed growing in some tropical countries (Hidayat, 2002; Fakhrudin, 2006).

Plant species of this genus have also been used for many decades in folk medicine as antimalaria, antibacteria, anti-oxidant, antifungi, antiimflamation and anticancer (Habtemariam, and Angela, 2000; Suksamran *et al.,* 2004, Begum *et al.,* 2007; Sukanya *et al.,* 2009; Chakra-varty *et al.,* 2011; Amatya and Tuladhar, 2011).

Dung et al., (1998) reported that the stem and leaf oils of *E. coelestinum* L., contains methyl chavicol, bornyl acetate, camphene and cis-cadin-4-en-7-ol (Dung *et al.,* 1998). Chrystomo *et al.,* (2011) reported that the methylripariochromene-A from *E. riparium* was only found in leaf, and the highest content of methylripario-chromene-A in benzene extract of *E. riparium* leaf was in those originated from Menoreh Mountain in Samigaluh, compared to those originated from Tawangmangu Karanganyar and Merapi Mountain in Kaliurang. Yunita *et al.,* (2009) reported that leaf extract of *E. riparium*, which contained saponin, tannin, quinon and steroid and this plant showed cytotoxicity on *A. aegypti* larvae and significant effect on percentage of pupae's development.

Other studies reported that *E. riparium* had alelopathy effect towards population of weeds *Galinsoga ciliata* Raf. and *Galinsoga parviflora* Cav. The water extract of *E. riparium* can inhibit the germination of seeds and the growth of radicule and plumulae of *Galinsoga ciliate* Raf. and *Galinsoga parviflora Cav*. (Kunwar 2003; Rai and Tripathi (2005).

Fakhrudin (2006) isolated methyl-ripariochromene-A from chloroform extract *of E. riparium* and this compound showed had cytotoxic activity against HeLa and Vero cell line.

In this research, the antiproliferative activity of benzene extract of *E. riparium* leaves from Menoreh Mountain in Samigaluh was examined *in vitro*, against C2C12 and MKN45 cell line.

MATERIALS AND METHODS

Preparation of *E. Riparium* Benzene Extract

E. riparium Reg. plants were collected from Menoreh mountains in Samigaluh. The plant was identified by Taxonomy Laboratorium, Gadjah Mada University. The leaves were washed, dried and chopped finely using a blender. Dried materials were exhaustively extracted with benzene maseration. The benzene extract was filtered and concentrated using a rotary evaporator and then evaporated to dry.

Preparation of Cell Line

MKN45 cell line (*human gastric cell line*) was obtained from Yamamura, Ph.D, Laboratorium Onkologi, Kawasaki Medi-cal School, Jepang. MKN45 cell line was grown on Dulbecco's Modified Eagle Media (DMEM, Sigma) containing 10% v/v Fetal Bovine Serum (FBS) (Sigma) and 1% v/v kanamicin (Sigma). C2C12 cell line (*adherent mouse myoblast cell line*) was obtained from Laboratory stock of Department Moleculer and Developmental Biology, Kawasaki Medical School, Japan. C2C12 was grown on DMEM (Sigma) medium containing 10% v/v Fetal Bovine Serum (FBS) (Sigma) and 1% v/v kana-micin (Sigma). The cultures were main-tained at 37^{o}C in humidified atmosphere of 5% CO_2.

In vitro assay for antiproliferative activity

The cell suspension $4.0x10^3$ cell/ml (100µl) was plated into 96 well microplate (Nunc, Germany) and treated with differ-

rent concentration of benzene extract isolated from *E. riparium* leaves, in a serial dilution (500, 250, 125, 62.50, 31.25 dan 15.625, 7.8125 µg/ml). Following treatment, plates were incubated in CO_2 incubator at 37°C for 72h. Medium was removed by aspirator and add medium with 10µl cell proliferation reagent WST-1 for 1h, 2h and 4h and incubated in CO_2 incubator at 37°C. The absorbance was read at wavelength of 450 nm using ELISA reader type Varioskan Flash (Thermo scientific). The percentage cellular viability was calculated with appropriate control taken into account. The concentration which inhibited 50% of cellular growth (IC_{50}) was determined. The inhibitory rate of cell proliferation was calculated by the following formula:

$$\text{Growth inhibition } (\%) = \frac{\text{OD Control-OD treated}}{\text{OD Control}} \times 100$$

RESULTS AND DISCUSSION

In this study, toxicity data are expressed as IC_{50}, a concentration of extracts that causes 50% cell survival and was obtained by plotting the percentage of survive cell versus concentration of benzene extract samples (Francoeur and Assalian, 1996). The extract that gave a IC_{50} value of 1000 µg/ml or less was considered to have chemoprenventif activities (Doyle and Griffiths, 2000).

The results showed that the benzene extract of *E. riparium* possessed antiproliferative effect against cell C2C12 and MKN45 cell line with IC_{50} values of 119.14 µg/ml (1h), 100.74 µg/ml (2h) and 70.65 µg/ml (4h) for C2C12 cell line and IC_{50} values of 201.74 µg/ml (1h), 135.06 µg/ml (2h) and 124.54 µg/ml (4h), for MKN45, respectively. It indicated that benzene extract of *E. riparium* leaves possessed potential antiproliferative effect against C2C12 and MKN45 cell line. The cytotoxicity result indicated time- and dose-dependent concentration of the extract.

The activities of these extract against C2C12 and MKN45 cell line might be due to the presence of highly complex compounds that are present in *E. riparium*. Different compound might influence different biochemical processes or stages in different manners.

Several study have reported that Genus Eupatorium contains sesquiterpene lactones, diterpene lactones, flavonoid, terpenoid and sterol. Sesquiterpene lactones and diterpene lactones showed cytotoxic activity on cell lines (Suksamran *et al.,* 2004; Huo *et al.,* 2004; Shen *et al.,* 2005; Zhang *et al.,* 2008).

Sesquiterpene lactones and diterpene lactones isolated from *E. kiirunense* showed cytotoxic activity on HeLa cell line (Shen *et al.,* 2005) and eupanilia C isolated from *E. lidleyanum* had cytotoxic activity against p-338 and A-549 cell line (Huo *et al.,* 2004).

Chemical compounds composition of *Chromolaena odorata* (synonym; *E. odoratum*) extract consists of flavonoids, saponins, tannins and steroids. *Chromolaena odorata* extracts revealed to have antibacterial activities, ability to inhibit the growth of *Bacillus subtilis*, *Staphylococcus aureus*, as well as to have antiprotozoa activity. Cytotoxicity assays against parasites indicated that it can reduce the number of parasites *Trichomonas vaginalis* and *Blastocystis hominis* (Vital and Rivera, 2009).

Chrystomo *et al.,* (2011) reported that the methylripariochromene-A have been isolated from benzene extract of *E. riparium*. Fakhrudin (2006) showed that the methylripariochromene-A isolated from chloroform extract of *E. riparium* had cytotoxic activity toward Hela and Vero cell line with IC_{50} of 58.32 µg/ml, and 80.95 µg/ml, respectively. Other studies have reported that methylripariochromene-A had antifungal activity (Sharma *et al.,* 1998). Methylripariochromene-A isolated from of *E. riparium* has antifungal activity towards pathogenic fungi *Colletotrichum gloeosporioides* (Bandara *et al*., 1992).

Studied by Hidayat (2002) reported that hexane extract of *E. triplinerve* had cytotoxic activity against myeloma cell line with ED_{50} of 5.85 µg/ml using *Brine Shrimp Lethality Test*. Therefore, the presence of the methylripariochromene-A, sesquiterpene lactones, diterpene lactones, flavonoid, terpenoid and sterol could be assumed to be responsible for the antiproliferative activities of benzene extract of *E. riparium* in this study.

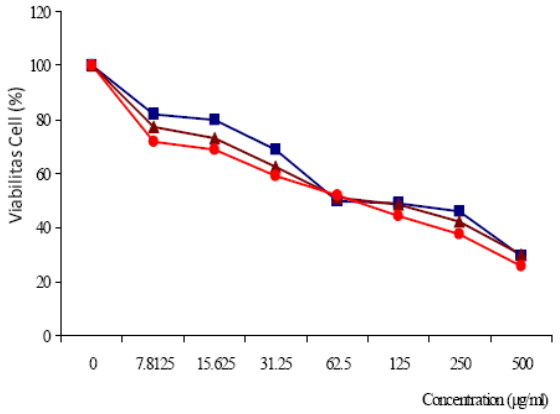

Figure 1. Correlation survival cell (%) of C2C12 cell line with extract concentration (µg/ml) after incubation for 72h at 37^{O}C with 5% CO_2. and after addition WST-1reagent for 1h (■- blue), 2h (▲-brown) and 4h (●-red).

Several study have reported many compounds from herbal or compounds from extract to have different cytotoxicity activity on different cell line. Arkadiusz *et al.,* (2001) reported that quercetin and DMSO modulated and changed Bcl-2 gene expression (Apoptosis regulating proteins) during myogenesis on C2C12 cell line.

Shao *et al.,* (2005) reported that Arsenic trioxide (As2O3), a major ingredient of traditional Chinese medicine showed cytotoxic activity in MTT assay and induced apoptosis of human gastric carcinoma cells (MKN45), indicated by the presence of cell shrinkage, membrane blebbing, fragmentation of nuclei, and formation of apoptotic bodies on MKN45 and other study by Yu *et al.* (2006) showed that antitumor effect of Chinese compound Jinlongshe (JLS) granules on sarcoma 180 and MKN-45 human gastric cancer cell lines in vivo.

In this study, the ability of benzene extract to inhibit proliferation of C2C12 and MKN45 cell line was estimated by analyzing its effect on the growth of the cells. The growth of the untreated (control) and treated cell line after incubation for 72 hours was photographed using a phase contrast microscope (data not shown). In the untreated cells, after 72h of incubation, cells were growing normally as indicated by the presence of formazan dye formed. The higher the extract concentration, the lower the formazan dye. The formation of formazan dye directly correlated to the number of metabolically active cells in the culture (Francouer, and Assalian. 1996). It is indicated that benzene extract of *E. riparium* leaves was proved to possess antiproliferative properties against C2C12 and MKN45 lines tested. Therefore, it may have potential as a chemotheraupetic agent since it has IC_{50} values less than 1000 µg/ml (Doyle and Griffiths, 2000).

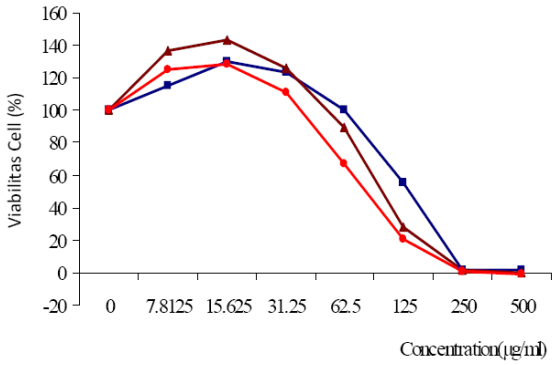

Figure 2. Correlation survival cell (%) of MKN45 cell line with extract concentration (µg/ml) after incubation for 72h at 37^{o}C with 5% CO_2. and after addition of WST-1reagent for 1h (■- blue), 2h (▲-brown) and 4h (●-red).

Further investigation is needed to know about inhibitory mechanism on C2C12 and MKN45 cell line.

CONCLUSION

The benzene extract of *E riparium* leaves possessed potential antiproliferative

activity againts C2C12 and MKN45. This plant have potential as anticancer agent.

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