Cytotoxic activity of *Combretum quadrangulare* leaf extracts on HepG2 cancer cell line

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Abstract

Studies on anticancer using plant extracts/compounds are promising. However, information regarding in vitro cytotoxicity is incompleted. Herein, this study aims to evaluate the cytotoxicity potentials of hexan:ethyl acetate (50:50, v:v) and ethanol extracts of Combretum quadrangulare leaves on HepG2 cancer cell line. Using cell viablity MTT assay, hexan:ethyl acetate and ethanol extracts were defined as potential cytotoxicity with IC50 at 38 and 47 µg/mL respectively. In addition, by microscopy observation, we found that morphology of the cells apparently change in a dose and time dependent manner. The data showed in this study shed new light for further investigation of the anticancer effect of C. quadrangulare extracts and its compounds

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combretum quadrangulare, extract, cytotoxicity, HepG2, MTT.

1. Introduction

Cancer is a class of diseases characterized by out-of-control cell growth. Nowadays, there are over 100 different types of cancer. Lung, prostate, colorectal, stomach, and liver cancer are the most common types of cancer in men, while breast, colorectal, lung, uterine cervix, and stomach cancer are the most common among women (WCRF). Studies on anticancer using plant extracts and compounds are promising. In eastern Asia, plants are widely being used for traditional medicine.

Combretum species are widely used as medicine for the treatment of some diseases such as hepatitis, malaria, respiratory infections, and even cancer in eastern Asia [3]. *Combretum quadrangulare* is an indigenous tree in eastern Asia. In Vietnam it is commonly known as "Tram bau", widely contributed and used as traditional medicine as an antipyretic, antidysenteric, anthelmintic agent [4]. Several Cycloartane Triterpenes have been isolated and shown to have biological activity on liver protection and cytotoxicity of some cancer cell lines [1, 2, 3, 4, 7, 8]. However, the activity of *C. quadrangulare* on HepG2 cells, a liver cancer cell line, is not fully understood. In this study, hexan:ethyl acetate (50:50) and ethanol extracts of *Combretum quadrangulare* leaves were tested on HepG2 cells for their cytotoxic possibilities.

2. Materials and methods

Cell line. HepG2 cell line was a gift of Dr. Pham Van Phuc, Lab of Stem Cells (National University in Ho Chi Minh city).

Complete growth medium. High glucose Dulbecco's Modified Eagle's Medium (DMEM, GIBCO) supplemented with 10% Fetal Bovin Serum (FBS, GIBCO) and 100 U/mL penicillin, 100 µg/mL streptomycin (Mekophar). Complete growth medium should be pre-warmed before use by placing into a water bath (Memmert) set at 37^{o}C ± 1^{o}C for 30 minutes.

Combretum quadrangulare leaves extracts and standards. Hexan:ethyl acetate (H:Ea, 50:50) and ethanol (EtOH) extracts were prepared from *C. quadrangulare* leaves. Doxorubicin (Fresenius Kabi) and dimethyl sulfoxide (DMSO, Fisher chemical) was used as positive control and negative control respectively.

Chemicals. 0.25% (w/v) trypsin (Sigma) - 1mM Na_2-Ethylenedia-minetetraacetic acid (EDTA, Thermo) was used for cell detaching and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) for cell viability assay. Detergent Reagent contained 10% (w/v) sodium dodecyl sulfate (SDS, Merck) and 10mM HCl (Merck). 0.4% (w/v) trypan-blue for cell staining was

purchased from Biobasic. Steriled phosphate buffer saline (PBS) consisted of 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4, pH 7.4 (all components were purchased from Merck).

Cell culture. HepG2 cell line was seeded in complete growth medium at 1 x 10^4 cells/cm^2 density and incubate at 37°C and 5% CO_2.

HepG2 growth curve. In order to assess growing possibility, HepG2 cells were seeded to 96-wells plate at 5x10^3 cells/cm^2 density and incubate at 37°C and 5% CO_2. Count the cells with a hemacytometer everyday up to 7 days of culture. The experiment was done in triplicate.

*Cell viability assay***.** Dissolve *Combretum quadrangulare* extracts in DMSO at 10 mg/mL concentration. The extracts was dilluted in culture medium at 100, 50, 25, 12.5, 6.25, 3.125, 0 µg/mL concentration. Stock of positive control containing 2mg/mL Doxorubicin in 20% DMSO were dilluted in culture medium at corresponding concentration to dilluted *C. quadrangulare* extracts. DMSO was used as negative control. In addition, culture medium without cells was used as Blank. Preparation and usage of the standards were done according to ISO 10993-5 and 12 [5, 6]. Seed the cells to 96-wells plate at 1 x 10^4 cells per well, incubate at 37°C and 5% CO_2. After 24 hours, replace the old medium by 100 µL of dilluted *C. quadrangulare* extracts, Doxorubicin or DMSO. Incubate samples at 37°C and 5% CO_2. Cell morphology was observed after 24, 48, 72 hours of treatment under inverted microscope. After 72 hours of treatment, add 10 µL of MTT (5 mg/mL) into each well and incubate at 37 °C and 5% CO_2 for 3.5 hours to produce purple formazan. Add 80 µL Detergent Reagent and incubate at 37°C and 5% CO_2 for 16-18 hours or overnight to dissolve crystal. Samples were read at 595nm by ELISA Reader for their absorbance. Cell death (% Inhibition) was estimated by the following formula:

$$\% \text{ Inhibition} = 100\text{-}100*(A_{Sample} - A_{Blank})/(A_{DMSO} - A_{Blank})$$

Where,

A_{Sample}: Absorbance of Sample at 595 nm

A_{DMSO}: Absorbance of Negative control at 595 nm

A_{Blank}: Absorbance of Blank at 595 nm.

To define whether the extract was considered as potential cytotoxicity, International Standard - ISO 10993-5 [5] was applied.

*Statistical analysis***.** Statistical analyses were performed using GraphPad Prism version 5.0 software.

3. Results and Discussion

3.1. Growth curve of HepG2 cell line

HepG2 cells were grown at density of 5x10^3 cells/cm^2. Our data showed that HepG2 cells strongly grow in our set up condition and that more than 2 x 10^5 cells were obtained after 7 days of culture (Fig 1).

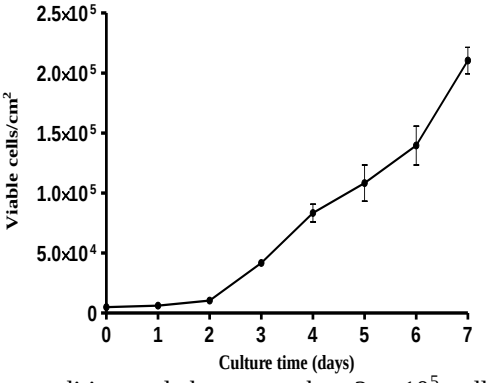


Figure 1. HepG2 growth curve during 7 days**.** The bars show mean ± SD

3.2. Cytotoxic activity

*Morphological assessment***.** As showed in table 2, with the extracts of *C. quadrangulare* leaves treatment, morphology of HepG2 cell line changed in a dose and time dependent manner. At concentration of 100 µg/mL of H:Ea extract, all cells changed from adhesion-shape to round-shape after 24 hours of treatment, but at concentration of 100 µg/mL of EtOH extract, there was quite rare round-shaped cells. At concentration of 50 µg/mL of H:Ea extract, density of round-shaped cells increased according to treatment time. At concentration of 50 µg/mL of EtOH extract, HepG2 morphology was likely unchanged.

Cytotoxic activity evaluation using MTT assay.

In contrast to EtOH extract, H:Ea extract were expressed higher cytotoxic activity against HepG2 cell line. At 3.125–25 µg/mLconcentration, H:Ea and EtOH extracts caused cytotoxicity to less than 15% of the cells. At 50 µg/mL concentration, EtOH extract caused 54% cells dead, H:Ea extract up to 74%. At concentration of 100µg/mL extracts, EtOH caused 60% cell dead, H:Ea extract up to 96% and this value was higher than inhibition percentage value of Doxorubicin (87%) at equivalent concentration. According to ISO 10993-5, IC_{50} value of >27 µg/mL and >31 µg/mL concentration of H:Ea and EtOH extract, respectively, was considered as potential cytotoxicity.

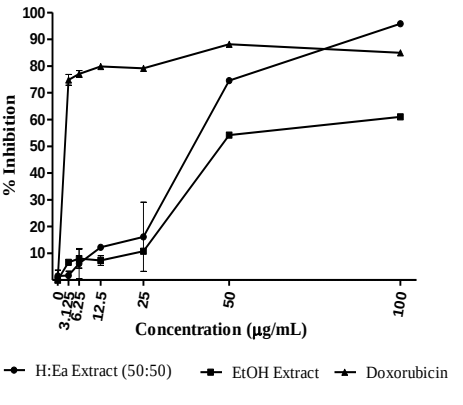
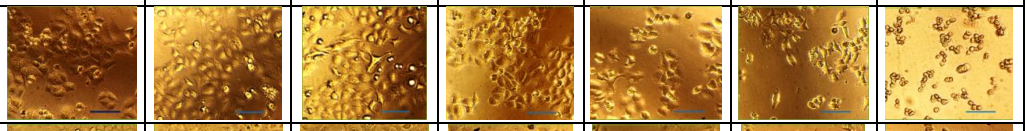
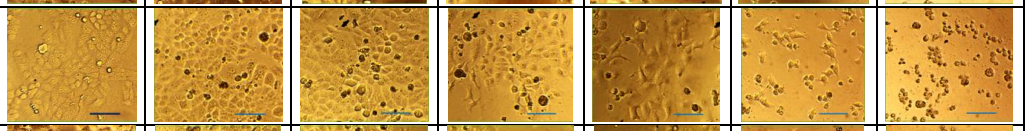
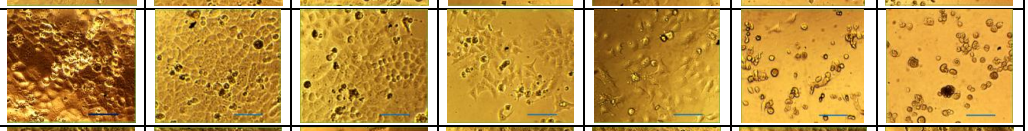
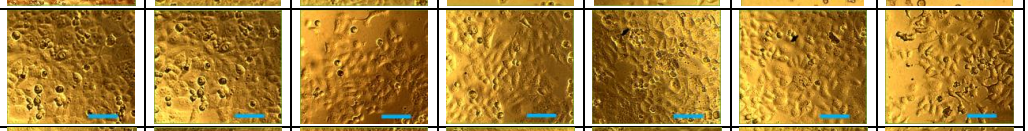
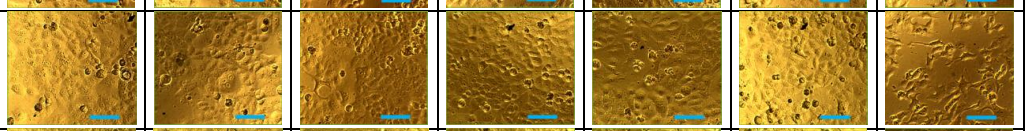
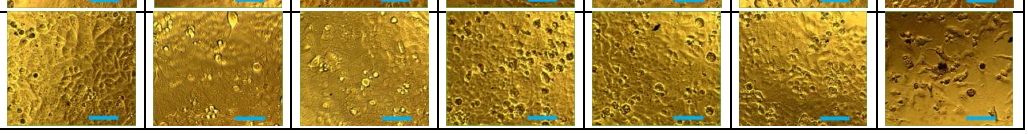


Figure 2. Effect of Combretum quadrangulare extracts on HepG2 cell line. Each value represents the mean of the three wells ± standard error. Statistical significant was set at 0.05 using one-way ANOVA method, P<0.005 (**)

Table 1. IC_{50} value of *C. quadrangulare* extracts and Standard using graphic method.

Extracts and Standard	IC_{50} (µg/mL)
Hexan:ethyl acetate	38
Ethanol	47
Doxorubicin	2

Table 2. Figures of hepG2 morphology at different concentrations of H:Ea and EtOH extracts and Doxorubicin after 24, 48, 72 hours treatment (100X). Scale bar, 100µm.

Extracts	Treatment time (hours)	Concentrations (µg/mL)						
		0	3.125	6.25	12.5	25	50	100
H:Ea (50:50)	24							
	48							
	72							
EtOH	24							
	48							
	72							

4. Conclusions

C. quadrangulare H:Ea and EtOH extracts possessed an inhibiting effect on the growth of HepG2 cell line. The extracts induce cell morphology from adhesion-shape to round-shape in a dose and time manner. Our preliminary data show that C. quadrangulare extracts can be consider for further investigation on its anticancer treatment.

Today, researching on plant extracts and compounds are promising way in anticancer treatment. The present study was an initial step of identifying the cytotoxicity effect of Combretum quadrangulare extracts on HepG2 cell line and in furtherance of its compounds in various in vitro studies for anticancer treatment.

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