Full Length Research Paper

An evaluation of the anticancer activity in *Hopea odorata* extracts

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Possible anticancer characteristics of *Hopea Odorata* extracts were investigated by cell proliferation and viability studies of cells in culture. The mechanism of action was studied by determining the rate of apoptosis and expression of protein involved in signal transduction. The result indicated that the butanol extract of *H. Odorata* had selective inhibition to both Hep G2 and Chang cells with IC50 of 20.14 and 377 µg/ml, respectively. Growth inhibition by the extract showed an increased of apoptosis at concentration of 25 µg/ml. Cell study demonstrated morphologic changes characteristic of apoptosis such as chromatin condensation and fragmentation, as well as formation of apoptotic bodies. However, MAPK kinase signal transduction pathway indicated no difference in ERK1 and ERK2 expression level after exposure at varying time. P53 protein level also showed no changes in expression compared to control. In conclusion, the increase in apoptosis observed was not due to changes in MAPK pathways involving ERK1, ERK 2 and p53 but may involve other pathway, which require further investigation.

Key words: Antiproliferation, *Hopea Odorata*, apoptosis.

INTRODUCTION

*Hopea Odorata* belongs to the Dipterocarpaceae family, locally known as Merawan siput jantan. It can grow up to 120 feet to produce good quality timber. The wood of *H. Odorata* varies in colour from a very pale yellow, or white to brown when first cut and characteristically darkens to a brownish or yellowish-brown colour after more or less prolonged exposure to the air. The dammar of this tree is said to have medicinal property used in treating sores and wounds (Burkill, 1935). Phytochemistry studies reported that the heartwood of *H. Odorata* contain certain types of phenolic compounds (Coggon et al., 1964). These polyphenols are reported useful as antioxidants, antimitagens, scavengers of free radicals and therefore have implications in the prevention of pathologies such as cancer and cardiovascular disease (Scalbert et al., 2005). In the quest for anticancer agents from natural sources, thousands of natural compounds were screened using both in vitro and in vivo methods (Balis, 2002). Due to the potential ability of these polyphenols as probable antitumour/antimutagen factor, the study was conducted to examine the cytotoxic and apoptotic effect of crude extract (CE) of *H. Odorata* using in vitro methods involving liver cancer cell lines (Hep G2) and a normal liver cell line (Chang cell).

MATERIALS AND METHODS

Chemicals and reagents

Tissue culture disposables and growth media for cell culturing were purchased from Nalgene and Flow Lab, respectively. Tetrazolium compound (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt; MTS) and an electron coupling reagent (phenazine methosulfate; PMS) and 5-Bromo-2-deoxy-Uridine (BrdU) were purchased from Promega (USA) and Boehringer Mannheinn (Germany), respectively. Three *H. Odorata* extracts tested were prepared according to the Figure 1. All the plant extracts were dissolved in dimethyl sulfoxide (DMSO) and diluted with culture medium. The final concentration of DMSO used was adjusted to 1% (v/v), the concentration used in control cell. All other chemicals were purchased from Sigma Chemical Co.
**Cell culture**

The hepatoma cell line, Hep G2 and Chang normal liver cell line were obtained from American type cell collection (ATCC), Rockville, MD and was cultured in Earle’s minimum essential medium (MEM) containing L-Glutamine and sodium bicarbonate and supplemented with 10% fetal bovine serum in 5% CO₂ incubator at 37 °C. Stock was allowed to grow to 80 to 90% confluence in T-75 flask. Culture medium was changed every two to three days. Cell viability was monitored using trypan blue exclusion test.

**In vitro cytotoxicity assay**

The proliferation assay was performed in 96 well flat bottom micro-titer plate at a plating density of $2 \times 10^4$ cell/well in a total volume of
100 ml of medium. Both Hep G2 cell and Chang cell were incubated at 37°C for 48 h with and without the test compounds at the following final concentration; 0, 10, 25, 50, 70, 100, 250, 500, 750 and 1000 µg/ml. Cells grown in a micro titer plate (MTP) were incubated with BrdU (10 µmol/l) for 18 h. The cells were then fixed with ethanol/HCl followed by incubation with nucleases to partially digest the DNA. The cell proliferation was then measured by quantitating BrdU incorporated into the newly synthesized DNA of replicating cells (Gratzner, 1982). Incorporated BrdU was detected with the monoclonal anti BrdU-POD. Fab fragments and the bound conjugate was visualized with the soluble chromogenic substrate 2,2'-azino bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and measured using an ELISA reader at wavelength of approximately 490 nm.

Cellular growth in the presence and absence of plants extract was also determined by using the MTS/PMS assay (CellTiter 96 AQuesus Non-Radioactive Cell Proliferation Assay, Promega, USA), according to the manufacturer’s protocol. This assay is a colorimetric method, in which MTS is bioreduced to a formazan product that is soluble in tissue culture medium by dehydrogenase enzyme that is found in metabolically active living cells (Barltrop et al., 1991). The intensity of formazan product as measured at absorbance wavelength of 490 nm absorbency, is directly proportional to the number of living cells in cultured and is the measure of cell viability (Mossman, 1993). IC50 values were expressed as microgram of compound concentration per millilitre that caused a 50% growth inhibition as compared to controls (cell growth in the absence of extract).

Apoptosis assay
Apoptosis is characterized by cleavage of the genomic DNA into discrete fragments prior to membrane disintegration. In this study, Cellular DNA fragmentation ELISA purchased from Boehringer Mannheim was used to determine the apoptotic cell death. 2 × 10⁴ BrdU labelled cells in the presence and without H. odorata extract, incubated for 24 h. At the end of the incubation, cells were centrifuged and cellular lysate was analysed for apoptosis.

Fluorescence microscopic analysis
Hep G2 cell (7 × 10⁵ cell) was cultured in EMEM medium supplemented with 10% fetal bovine serum in petri dish. After 24 hours of incubation, the medium was replaced with EMEM containing fetal bovine serum with 25 µg/ml H. odorata butanol extract. The cells were incubated for 4 h and the fixed with 1% cold formaldehyde in PBS for 30 min. The cell was then washed with PBS and stained with 1 ml 10 µg/ml Propium iodide. The specimens were analysed by florescence microscopy.

Differential staining of cells
Cell cultured on cover slips and exposed to H. odorata butanol extract for 24 h were fixed in 100% methanol. The nuclei were then stained with acid dye and finally, the cytoplasm was stained using basic dye. The morphological appearance of the cell was viewed under a light microscope.

Protein extraction and western blot analysis.
8.5 × 10⁶ cells were cultured and after 24 h incubation, the medium was replaced with a fresh medium containing 25 µg/ml butanol extract of H. odorata. Cells were incubated at different time duration of 0, 2 h, 6 h, 12, 18 and 24 h. Cells were then trypsinized and lysated, supernatant was kept at 80°C until used. 60 µl protein was boiled at 95°C for 5 min and were resolved on 10% SDS-polyacrylamide gel, transferred onto nitrocellulose and immunoreacted with primary antibodies, followed by a 1 h incubation with secondary antibodies conjugated with alkaline phosphatase. The primary antibodies were mouse monoclonal anti-human p53, ERK 1 and ERK 2 (Pharmagen). Visualization was performed using chemiluminescence detection (Pierce).

RESULTS AND DISCUSSION

The viability assay results are concurrent with the proliferation assay in which only butanol extract of H. odorata showed anti proliferation effect. Figure 2 represents the results of anti proliferation for 48 h as measured by BrdU assay in an in vitro system. Results are expressed as the percentage of absorbency of cells incubated with different doses of extracts against control. As shown in Figures 2 and 3, only butanol extract showed significant growth inhibition, IC50 (Hep G2) is equal to 20.14 µg/ml and at the same time need a higher concentration (IC50 = 377.60 µg/ml) to produce the same effect in normal Hepar cell, Chang. Those data suggested that the Butanol extract have selective toxicity towards liver tumour cells. Two other extracts did not show any significant inhibition. Hence, only butanol extract was used for further studies.

Cellular DNA fragmentation ELISA revealed that anti proliferation demonstrated by butanol extract via apoptosis pathway. Figure 4 shows detection of nucleosomes in the cytoplasmic fractions at different extract concentration after 24 h incubation. Fluorescence microscopy analysis revealed that the Butanol extract of H. odorata induced apoptosis and caused morphological changes of cells undergo apoptosis. Cell undergoing apoptosis process showed sequence of morphological modification that include nucleolus and cytoplasm condensation with a pronounced decrease in cell volume, chromatin condensation and fragmentation, plasma membrane blebbing and degeneration of the nucleus into membrane bound apoptotic bodies (Steller, 1995). For differential staining, the result demonstrated cells undergo nuclear shrinkage, cytoplasmic constriction and formation of apoptotic bodies as shown in Figure 5. Signal transduction has been the target for anti cancer by inhibiting phosphorylation process (Seymour, 1999). One of the pathways is MAP kinase which is involved in extracellular signal regulation and ERK 1 and ERK2 are among the unique component in this transduction pathway (Willbacher et al., 1999). While the p53 is the nuclear phosphoprotein involved in stimulating apoptosis directly by binding and act on several protein in variety pathway including C-Abl, basal transcription factor and so on (Oliner, 1992). Western blot Analysis demonstrated (Figure 6) that after treatment with 25 g/ml H. odorata, the level of p53 protein was apparently unchanged with
Effect of *Hopea Odorata* extracts on Hep G2 cells proliferation using BrdU labelling assay

![Graph showing inhibition percentage vs concentration in the presence of plant extracts at different concentration after 48 h incubation.](image)

**Figure 2.** Percentage of cell proliferation, measuring with BrdU labelling assay in the presence of plant extracts at different concentration after 48 h incubation.

Effect of *Hopea Odorata* extracts on Chang cells proliferation using BrdU labelling assay

![Graph showing inhibition percentage vs concentration in the presence of *Hopea odorata* extracts at different concentration.](image)

**Figure 3.** Percentage Chang cell inhibition after 48 h incubation in the presence of *Hopea odorata* extracts at different concentration.

respect to the level found in the untreated cells. ERK 1 and ERK 2 expression also remained unchanged after having treatment with *H. Odorata* from 0 hour up to 24 hours. The apoptosis induced by *H. Odorata* butanol
Figure 4. Measuring apoptosis with the cellular DNA fragmentation ELISA BrdU-labelled Hep G2 cells were cultured in the presence of *Hopea Odorata* Butanol extract for 24 h. After incubation, the lysates were tested by ELISA.

Figure 5. Morphological features of *Hopea Odorata* butanol extract treated Hep G2 cells (25 µg/ml) after 24 h incubation examined by differential staining. (a) and (b) showed nuclear shrinkage and cytoplasmic constriction, (c) showed formation of apoptotic bodies.

Extract in Hep G2 seem to be determined by other mean of mechanism. In this case, apoptosis is not accompanied by the increments in p53. Thus, the probability of *H. Odorata* being involved in yet other signal transduction pathway leading the proliferation pathway of cell growth and further studies will be needed.

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Figure 6. Western blot Analysis of (a) ERK 2, (b) ERK 1 & ERK 2, and (c) p53. Extracts were prepared from cells as described in methods. Cells were exposed to 25 µg/ml plant extract at different time duration (0, 1, 2, 6, 12, 18, and 24 h. 60 µg of protein were submitted to western blot analysis and assayed for particular proteins expression.

REFERENCES


