Malaysian Journal of Medicine and Health Sciences Vol. 1 (2) June 2005 : 105 - 110

Effects of *Catharanthus roseus*, *Kalanchoe laciniata* and *Piper longum* Extracts on the Proliferation of Hormone-dependent Breast Cancer (MCF-7) and Colon Cancer (Caco2) Cell Lines

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ABSTRACT

Introduction: This research was conducted to investigate the effects of *Catharanthus roseus* (Kemunting Cina), *Kalanchoe laciniata* (Setawar Kampung) and *Piper longum* (Kadok Kampung) on the *in vitro* proliferation of hormone dependent breast cancer (MCF-7) and colon cancer (Caco2) cell lines. **Methodology:**The effects of *Catharanthus roseus, Kalanchoe laciniata* and *Piper longum* extracts (hexane, chloroform, ethyl acetate and methanol extracts) on the cytotoxicity of MCF-7 and Caco2 cell lines were measured using (3-[4,5-dimethylthiazol–2-yl]-2,5-diphenyltetrazoliumbromide) (MTT) assay. **Results:** *Kalanchoe laciniata* hexane extract showed significant inhibitions on MCF-7 carcinoma cell lines proliferation with IC_{50} value of 75.7 μg/ml. *Catharanthus roseus* extracts (hexane, chloroform and ethyl acetate) inhibited MCF-7 cells proliferation at concentrations of 80, 70 and 90 μg/ml, respectively. **Discussion:** IC_{50} value of all extracts of *Piper longum* and *Kalanchoe laciniata* chloroform, ethyl acetate and methanol extracts on the proliferation of MCF-7 cancer cells could not be determined, as it did not demonstrate any appreciable inhibition on the cellular proliferation at the concentration tested. However, *Piper longum* chloroform and ethyl acetate extracts showed anti-tumourigenic effect against colon cancer (Caco2) with IC_{50} of 87 μg/ml and 20 μg/ml, respectively. *Kalanchoe laciniata* hexane extracts inhibited Caco2 cellular proliferation with IC_{50} value of 100 μg/ml. *Catharanthus roseus* chloroform and ethyl acetate extracts inhibited Caco2 proliferation at IC_{50} of 28.2 μg/ml and 74.1 μg/ml, respectively. **Conclusion:** From this study, it can be concluded that only hexane extract of *Kalanchoe laciniata* was effective against cellular proliferations of MCF-7 while *Piper longum* was more effective in inhibiting Caco2 proliferations. However, both the herbs were not so effective against MCF-7 and Caco2 cell lines compared with *Catharanthus roseus.*

Keywords: ***Catharanthus roseus*, *Kalanchoe laciniata*, *Piper longum*, cytotoxicity**

INTRODUCTION

Catharanthus roseus is from Apocynaceae Juss family. Its common names are Rose Periwinkle and Old Maid. In Malaysia, *Catharanthus roseus* is known as *Kemunting Cina*. It originated from Madagascar and India. *Catharanthus roseus* has green stem, oval leaf but is round at the end. It also has white or pink flowers. It usually grows wild or is planted as

an ornamental tree [1]. *Catharanthus roseus* leaves are used traditionally to treat diseases. The leaves are soaked in water, which is then drunk to ease menstrual pains. The leaf extract is applied to insect bites. It has been reported that this plant has been used in the treatment of diabetes, hypertension and cancer [1].

Kalanchoe laciniata or *Setawar Kampung* is from the crassulaceae family and can be found at village sites whether as a decorative plant or for medical purposes. Its stem is green, 30 cm high and 2-3 cm in diameter. The clump roots are white in colour. It also has thick green leaves with irregular borders. The young leaves have three branches. *Kalanchoe laciniata* has numerous traditional medical uses. The ground leaves of *Kalanchoe laciniata* are used to treat headache, swelling, pain and cancer. Its leaves are also used in lotion ingredients for chicken pox. The leaves are patched on the chest to treat cold and fever.

No studies have been done on *Kalanchoe laciniata*. However, many studies have been done on the *Kalanchoe* genus. Previous studies show moderate activity of *K. pinnata* aerial parts (used in inflammatory processes), for anti-malarial activity *in vitro* on *P. falciparum* and *P. vinckei petteri* [2]. The fatty acids present in *K. pinnata* may be responsible for its immunosuppressive effect *in vivo* [3]. It was found to cause significant inhibition of cell mediated and humoral immune responses in mice [4]. *K. brasiliensis* also proved to have anti inflammatory effect [5]. The anti-leishmanial effect of *Kalanchoe* is mediated by oxide intermediates. It significantly decreases the lesion size and the parasite load in BALB/c mice infected with *Leishmania amazonensis* [6]. Aqueous crude extracts (ACE) of *Kalanchoe* have been used as vaginal contraceptives [7]. Patuletin acetylphamnosides from *K. brasiliensis* are used as inhibitors of Human Lymphocytic Proliferative activity [8]. *K. laciniata* is said to have anticancer properties in Malay traditional medicine and is used to relieve headache, swelling, cough and irritated skin.

Piper longum is from the Piperaceae family. It is also known as Indian long pepper. However, its local name varies. Sometimes, *Piper longum* is known locally as kadok kampung, daun kadok, sirih dudok, akar bugu, kadok batu, mengkadak and kudak [1]. The entire plant parts of *Piper longum* are used including fruit, root and stem [1]. It grows on damp soil in secondary forests. It is also grown as a vegetable. The erect shrub has a thick and branched rootstock. Leaves are numerous, 6.3 to 9.0 cm, broadly ovate or oblong oval, dark green and shining above, pale and dull beneath. Fruits are present in a solitary, pedunculate, a fleshy spike 2.5 to 3.5 cm long, 5 mm thick, ovoid, oblong, erect, blunt, blackish green in colour and shining. Odour is aromatic and the taste is pungent.

Traditional medical use is by boiling water with *Piper longum*'s roots, and when taken, it is said to be a diuretic. To treat malaria, the water in which a bunch of kadok kampung leaves have been boiled is drunk. The drink is also used to treat cough, flu, lumbago, toothache and rheumatism [1].

Until today, there were very few scientific studies that have been documented on the effect of *Kalanchoe laciniata* and *Piper longum* on cancer cell growth. We therefore, investigated the cytotoxicity of these two plants and compared them with *Catharanthus roseus* from which Vinblastine and Vincristine (*Vinca* alkaloids) have been isolated, which is known worldwide for its anti-cancer properties.

MATERIAL AND METHODS

Three types of whole plants, *Catharanthus roseus*, *Kalanchoe laciniata* and *Piper longum* were collected at the village sites of Kampung Sawah, Pekan Nanas, Johor, Malaysia.

Sample Preparation

One hundred grams of leaves of each plant (*Catharanthus roseus*, *Kalanchoe laciniata* and *Piper longum)* were weighed, washed and cut into small pieces, separately. Then 500 ml of hexane solution was added into each type of leaves and blended. Three containers (bottles) were used to place and soak the mixture of the blended leaves and hexane for a day and the decoctions were filtered. The filtered extracts (filtrate) were each poured into a volumetric flask. 500 ml hexane was added to each residue separately, and soaked for another day before filtering again. A similar procedure was undertaken on the third day. Finally, the three filtrates were pooled and evaporated using the rotary evaporator. The final residue from hexane filtration was added with 500 ml of chloroform and soaked overnight. The procedure used for hexane extraction was repeated using chloroform, ethyl acetate and methanol. The concentrated crude extract obtained was weighed.

Cell Culturing

The carcinoma cell lines used were breast cancer hormone dependent (MCF-7) and colon cancer (Caco2). MCF-7 and Caco2 cell lines were obtained from American Type Culture Collection (ATCC), Rockville, Maryland, USA. The medium for both cell lines were Dulbecco's Modified Eagles Medium, DMEM (Gibco, USA). The cells were cultured with the medium supplemented with 10% of fetal calf serum and 1% of penicillin-streptomycin (Gibco, USA) using 25 cm^2 flasks (Nunc, Denmark), in a 5% CO_2 incubator (Sanyo, Japan) at 37°C.

Subculturing

The culture medium was replaced with new medium. The flask was rinsed with PBS-EDTA to wash the cells. Following that, PBS-EDTA was removed, 0.75 ml trypsin was added and the flask was incubated at 37°C and 5% CO_2 for 3-5 min; the flask was then 'knocked' to detach the cells from the bottom part of the flask, and trypsin was removed. Finally, 10-14 ml of the medium were added and divided into two parts; half of the culture was then transferred into a new flask. Cells were grown until they were confluent, harvested and the amount of viability cells was determined with trypan blue method and counted by using hemocytometer, and diluted with medium, yielding a concentration of 1 x 10^5 cells ml^{-1}. From this cell suspension, 100 ml was pipetted into each well of 96-well microtiter plate (Nunc, Denmark) and incubated for 24 hours in 5% CO_2 incubator (Sanyo, Japan) at 37°C.

Cell Proliferation Assay

Cells were grown in 96-well microtiter plate (Nunc, Denmark) in final volume of 100 ml culture medium per well. The cells were then treated with *Catharanthus roseus*, *Kalanchoe*

laciniata and *Piper longum* hexane, chloroform, ethyl acetate and methanol extract in different concentrations (5, 10, 20, 40, 60, 80, 100 µg/ml). The plate was then placed back in 5% CO_2 incubator (Sanyo, Japan) at 37°C for 72 hours. After the incubation period, 10 ml of the MTT labelling reagent (final concentration 0.5 mg/ml) (Roche Diagnostics, USA) was added to each well. The microtiter plate was then incubated for a further 4 hours at 37°C with 5% CO_2. Then, 100 µl of the solubilization solution (Roche, USA) was added into each well. The cells were allowed to stand overnight in the incubator at 37°C with 5% CO_2. Finally, cell viability was measured using an ELISA reader (El_x 800) at a wavelength of 550 nm.

% cytotoxicity = [OD sample (mean) / OD control (mean)] x 100%
OD = optical density

RESULTS

The absorbance value obtained from the ELISA reader was used to calculate the percentage of cytotoxicity of *Catharanthus roseus*, *Kalanchoe laciniata* and *Piper longum* hexane, chloroform, ethyl acetate and methanol extracts on hormone dependent breast cancer and colon cancer cell lines. IC_{50} values (concentration that inhibits 50% of cancer cell liability) for hormone dependent breast cancer are shown in Table 1 and for colon cancer in Table 2.

DISCUSSION

This study was undertaken with the purpose of determining the effects of *Catharanthus roseus* (kemunting cina), *Kalanchoe laciniata* (setawar kampung) and *Piper longum* (kadok kampung) extracts on the proliferation of hormone dependent breast cancer (MCF-7) and colon cancer (Caco2) cell lines. Each plant species underwent an extraction process that

Table 1. IC_{50} values of plant extracts against hormone dependent breast cancer (MCF-7) cell lines

Plants	*Catharanthus roseus* (µg/ml)	*Kalanchoe laciniata* (µg/ml)	*Piper longum* (µg/ml)
Hexane	80.0	75.7	-
Chloroform	70.0	-	-
Ethyl acetate	90.0	-	-
Methanol	-	-	-

Table 2. IC_{50} values of plant extracts against colon cancer (Caco2) cell lines

Plants	*Catharanthus roseus* (µg/ml)	*Kalanchoe laciniata* (µg/ml)	*Piper longum* (µg/ml)
Hexane	-	100	-
Chloroform	28.2	-	87.0
Ethyl acetate	74.1	-	20.0
Methanol	-	-	-

resulted in four different extracts, which were hexane, chloroform, ethyl acetate and methanol. MTT assay was used to determine the IC_{50} value of the samples at different concentrations (5-100 mg/ml).

From this initial screening study, *Kalanchoe laciniata* and *Piper longum* extract showed some degree of anti-proliferation properties against hormone dependent breast cancer (MCF-7) and colon cancer (Caco2) cell lines. *Kalanchoe laciniata* species exerted promising anti-tumor and immunostimulatory potential for breast cancer, while *Piper longum* was more likely to inhibit the proliferation of colon cancer cell lines.

By comparing the inhibitions of *Catharanthus roseus* extracts against both of the two plant species tested, *Catharanthus roseus* was still found to be the best chemotherapeutic agent. This was shown by the significant inhibition of its extracts against both MCF-7 and Caco2 cell lines. In the late 1950s and 1960s, a combined effort led to the isolation and structure elucidation of the active bis-indole alkaloids vinblastine, vincristine, leurosine and leurosidine. Two of them, vinblastine and vincristine, have been developed as commercial drugs. These compounds are antimitotics inhibiting cell growth, at least in part, by disrupting microtubules. As a consequence, the cell mitotic spindles are dissolute and the cells are arrested at metaphase.

Although natural antioxidants can be active themselves, most of them become effective in the presence of a synergist, such as α-tocopherol. Multiple purification of crude extracts of plant materials often results in failure to obtain a single strong antioxidant, due to the stability of the compounds in the crude extract form or due to the presence of a synergist. In addition, plants are a complicated mixture of numerous chemicals and interactions with their components may affect the effectiveness of the antioxidant. Though purification is suggested for further study, this effort may not be successful because the strong inhibitory activity of the crude extract may not be fully recovered in purified form. Thus, the significance of a combination of multiple compounds for anti-tumor promotion is again suggested.

In this study, four different solvents were used to obtain extraction based on polarity. Further screening test indicated some interference factors might frequently co-occur in the methanol extracts. Therefore, it is suggested that partitioning the extracts between ethyl acetate and water might be better. Hence, the anti-tumor promoting activity in the crude extracts may be enhanced or reduced with co-occurring factors acting additively, synergistically or antagonistically.

In this study, only leaves from the plants were used. The useful active components, which inhibit the carcinoma cells found in other parts of the plants, may not be found in the leaves. Therefore, some of the results showed negative inhibition of the cancer cell lines. It could be that the active compounds of the plants, which may act as an antioxidant, vary according to the different parts of the plants. A good example of this is Panax ginseng in which the whole plant or its saponin fractions are more active than the isolated compounds [9].

CONCLUSION

From this study, it can be concluded that only hexane extracts of *Kalanchoe laciniata* were effective against cellular proliferations of MCF-7 while *Piper longum* was more likely to be

effective in inhibiting Caco2 proliferations. However, these two herbs were not that effective against MCF-7 and Caco2 cell lines compared with *Catharanthus roseus,* from which vinblastine and vincristine (*Vinca* alkaloids), which are known worldwide for their cancer properties, were isolated.

ACKNOWLEDGEMENT

This project was funded by UPM (final year project grant, 2001) and IRPA grant No. 06-02-04-0050.

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